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Tiam1 (T lymphoma invasion and metastasis) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for RhoGTPases (e.g. Rac1) and is expressed in breast tumor cells (e.g. SP-1 cell line). Immunoprecipitation and immunoblot analyses indicate that Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo. In particular, the ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding. Biochemical analyses indicate that the 11aa sequence between aa717 and aa727 of Tiam1 (^{717}GEGTDAVKRS^{727}L) is the ankyrin binding domain. Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on RhoGTPases (e.g. Rac1).

Using an Escherichia coli-derived calmodulin-binding peptide (CBP)-tagged recombinant Tiam1 (aa393-aa728) fragment which contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (aa393-aa738) fragment and ankyrin in vitro. This Tiam1 fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin. Transfection of SP-1 cell with Tiam1 cDNAs stimulates (i)Tiam1-ankyrin association in the membrane projections, (ii) Rac1 activation and (iii) breast tumor cell invasion and migration. Co-transfection of SP1 cells with green fluorescent protein (GFP)-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1-ankyrin co-localization in the cell membrane and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes. These findings suggest that ankyrin-Tiam1 interaction plays a pivotal role in regulating Rac1 signaling and cytoskeleton function required for oncogenic signaling and metastatic breast tumor cell progression.

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INTRODUCTION

Members of the Rho subclass of the ras superfamily [small molecular weight GTPases, (e.g. RhoA, Rac1 and Cdc42)] are known to be associated with changes in the membrane-linked cytoskeleton (Ridley and Hall, 1992; Hall, 1998). For example, activation of RhoA, Rac1 and Cdc42 have been shown to produce specific structural changes in the plasma membrane-cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (Ridley and Hall, 1992; Hall, 1998). The coordinated activation of these GTPases is thought to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (Dickson and Lippman, 1995; Jiang et al., 1994; Lauffenburger and Horwitz, 1996).

Several guanine nucleotide exchange factors (GEFs - the dbl or DH family) have been identified as oncogenes due to their ability to up regulate Rho GTPase activity during malignant transformation (van Aelst and D'Souza-Schorey, 1997). One of these GEFs is Tiam1 (T lymphoma invasion and metastasis) which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior in vitro (Habets et al., 1994; Habets et al., 1995). This molecule is largely hydrophilic and contains several functional domains found in signal transduction proteins. For example, the C-terminal region of the Tiam1 molecule has a Dbl homology (DH) domain (Habets et al., 1994; Hart et al., 1991; Hart et al., 1994) and an adjacent pleckstrin homology (PH) domain which exists in most GEFs (Habets et al., 1994; Hart et al., 1991; Hart et al., 1994; Lemmon et al., 1996). In particular, the DH domain of these proteins exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (Hart et al., 1991; Hart et al., 1994). Tiam1 also contains an additional PH domain, a Discs-large homology region (DHR) (Habets et al., 1994; Pontings and Phillips, 1995) and a potential myristoylation site in the N-terminal part of the protein (Habets et al., 1994).

Overexpression of both N - and C-terminally truncated, as well as full-length, Tiam1 proteins induces the invasive phenotype in otherwise noninvasive lymphoma cell lines (Michiels et al., 1995). It is also well-established that Tiam1 is capable of activating Rac1 in vitro as a GEF and induces membrane cytoskeleton-mediated cell shape changes, cell adhesion and cell motility (Michiels et al., 1995; Woods and Bryant 1991; van Leeuwen et al., 1995; Nobes and Hall, 1995). These findings have prompted investigations into the mechanisms involved in the regulation of Tiam1. In fact, it has been found that addition of certain serum-derived lipids [e.g. sphingosine-1 -phosphate (S1P) and LPA] to T-lymphoma cells promotes Tiam1-mediated Rac1 signaling and T-lymphoma cell invasion (Stam et al., 1998). A Tiam1 transcript has been detected in breast cancer cells (Habets et al., 1995). Tiam1 is shown to function as a GEF in activating Rac1 signaling in breast tumor cells (Bourguignon, et al., 2000). The question of how this molecule is regulated in invasive and metastatic processes of breast cancer cells is addressed in the present study.

Ankyrin belongs to a family of cytoskeletal proteins that mediate linkage of integral membrane proteins with the spectrin-based skeleton in regulating a variety of biological activities (Bennett, 1992; Bennett and Gilligan, 1993; De Matteis and Morrow, 1998). Presently, at least three ankyrin genes have been identified: ankyrin 1 (ANK 1 or ankyrin R), ankyrin 2 (ANK 2 or ankyrin B) and ankyrin 3 (ANK 3 or ankyrin G) (Lambert, et al., 1990; Lux et al., 1990; Tse, et al., 1991; Otto, et al., 1991; Kordeli, et al., 1995; Peters, et al., 1995; Peters and Lux 1993). All ankyrin species (e.g. ANK 1, ANK 2 and ANK 3) are monomers comprised of two highly conserved domains and a variable domain. Both conserved domains are located in the N-terminal region and include a membrane-binding site [M. Wt. ≈89-95kDa, also called the ankyrin repeat domain (ARD)] (Lux et al., 1990; Davis and Bennet, 1990), and a spectrin binding domain (SBD) (M.Wt. ≈ 62kDa) (Platt,

et al., 1993). The striking feature shared by all three forms of ankyrins is the repeated 33-amino acid motif present in 24 contiguous copies within the ARD. The ARD of ANK 1, ANK 2 and ANK 3 is highly conserved. A number of tumor cells express ankyrin such as ANK1 and ANK3 (Bourguignon, et al., 1998b; Bourguignon, et al., 1999a; Zhu and Bourguignon, 2000). Most recently, we have found that ankyrin's ARD interacts with the adhesion molecule, CD44 and promotes tumor cell migration (Zhu and Bourguignon, 2000). In addition, the ARD domain (also referred to as cdc 10 repeats, cdc10/SW16 repeats, and SW16/ANK repeats) has been detected in a number of functionally distinct proteins participating in protein-protein binding and protein-DNA interactions (Lux, et al. 1990; Davis and Bennet, 1990).

In this study we have focused on the regulatory aspect of Tiam1-Rac1 signaling in metastatic breast tumor cells (SP-1 cell line). Our results indicate that Tiam1 interacts with ankyrin in vivo and in vitro. In particular, the ankyrin repeat domain (ARD) is directly involved in Tiam1 binding. Biochemical analyses show that the Tiam1 fragment (aa393-aa738) contains an ankyrin-binding site and competes for Tiam1 binding to ankyrin. Most importantly, the binding of ankyrin [in particular, ankyrin repeat domain (ARD)] to Tiam1 activates Rho-like GTPase such as Rac1. Overexpression of Tiam1 in SP-1 cells by transfecting Tiam1 cDNA induces Tiam1-ankyrin association in the cell membrane, Rac1 signaling and metastatic phenotypes. Both Tiam1-ankyrin interaction and tumor-specific behaviors are significantly inhibited by co-transfecting SP-1 cells with the Tiam1 (aa393-aa738) fragment cDNA and Tiam1 cDNA. Our observations suggest that Tiam1 interaction with ankyrin promotes RhoGTPase activation and cytoskeletal changes required for metastatic breast tumor cell invasion and migration.

BODY

MATERIALS AND METHODS

Cell Culture: Mouse breast tumor cells (e.g. SP1 cell line) (provided by Dr. Bruce Elliott, Department of Pathology, and Biochemistry, Queen's University, Kingston, Ontario, Canada) were used in this study. Specifically, SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen's University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (Elliott, et al., 1988). These cells were cultured in RPMI1640 medium supplemented with either 5% or 20% fetal calf serum, folic acid (290mg/L), and sodium pyruvate (100mg/L).

COS-7 cells were obtained from American Type Culture Collection and grown routinely in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1% glutamine, 1% penicillin and 1% streptomycin.

Antibodies and Reagents: For the preparation of polyclonal rabbit anti-Tiam1 antibody, specific synthetic peptides [≈15-17 amino acids unique for the C-terminal sequence of Tiam1] were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using an Advanced Chemtech automatic synthesizer (model ACT350). These Tiam1-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies. The anti-Tiam1-specific antibody was collected from each bleed and stored at 4°C containing 0.1% azide. The anti-Tiam1 IgG fraction was prepared by conventional DEAE-cellulose chromatography, respectively. Mouse monoclonal anti-HA (hemagglutinin epitope) antibody (clone 12 CA5) and mouse monoclonal anti-green fluorescent protein (GFP) antibody were purchased from Boehringer Mannheim and PharMingen, respectively. Escherichia coli (E. coli)-derived GST-tagged Rac1/Cdc42 and GST-tagged RhoA was kindly provided by Dr. Richard A. Cerione (Cornell

University, Itheca, NY) and Martin Dr. Martin Schwartz (Scripps Research Institute, La Jolla, CA), respectively. Mouse monoclonal erythrocyte ankyrin (ANK1) antibody was prepared as described previously (Bourguignon, et al., 1993a). Rabbit anti-ANK3 antibody was kindly provided by Dr. L. L. Peters (Jackson Laboratory, Bar Harbor, ME) (Peters, et al., 1995).

Cloning, Expression and Purification of GST-tagged Ankyrin Repeat Domain (GST-ARD) and GFP-tagged Spectrin Binding Domain (GFP-SBD) of Ankyrin: pGEX-2TK recombinant plasmid expressing GST-ARD (N-terminal portion of ankyrin, residues 1-834) was constructed as follows. Two pGEX-2TK recombinant plasmids pA3-79 (expressing epithelial Ank3 N-terminal 1-455 amino acids) and pA3-88 (expressing epithelial Ank3 N-terminal 317-834 amino acids) (Peters, et al., 1995) were kindly provided by Dr. L. L. Peters from Jackson Laboratory. The two plasmids were digested by EcoRI (one of pGEX-2TK vector cloning sites) and NheI (in ankyrin cDNA 1176 bp) sequentially. The digested products were run in 1% agarose gel and purified with Qiagen purification kit (Qiagen Inc., Chatsworth, CA). The larger cDNA fragment in pA3-79 digested products (containing the pGEX-2TK vector and ankyrin cDNA 1-1176 bp) and the smaller one in pA3-88 digested products (containing ankyrin cDNA 1176-2556 bp) were cut and purified. Then these two cDNA fragments were ligated and transformed to INVαF' competent cells. The obtained clones were sequenced to verify the correct generation of the full length ankyrin repeat domain (ARD).

Spectrin binding domain (SBD) cDNA of human erythrocyte ankyrin was cloned into the eukaryotic expression vector, GFPN1 (Clonetech) using the PCR-based cloning strategy. Ankyrin's spectrin binding domain cDNA was amplified by PCR with two specific primers (left, 5'-CGCTCGAGATGAAGGCTGAGAGGCGGGATTCC and right, 5'-ATAAGCTTCAGGGGCGTCGGGGTCCTTCT) linked with specific enzyme digestion site (Xho I and Hind III). PCR product digested with Xho I and Hind III was purified with QIAquick PCR purification kit (Qiagen). Ankyrin's spectrin binding domain cDNA fragment was then cloned into GFPN1 vector digested with Xho I and Hind III. The cDNA sequence was confirmed by nucleotide sequencing analysis. The GFP tagged spectrin binding domain (SBD) of ankyrin is expressed as a 89kDa polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses. The 89kDa GFP-SBD (but not ARD) displays specific spectrin binding property as described previously (Platt, et al. 1993). Subsequently, GFP-SBD was isolated from anti-GFP conjugated affinity columns and used in various in vitro binding experiments as described below.

Expression Constructs: Both the full-length mouse Tiam1cDNA (FL1591) and the NH_2-terminally truncated Tiam1cDNA (C1199) were kindly provided by Dr. John G. Collard (The Netherlands Cancer Institute, The Netherlands). Specifically, the full-length Taim1 (FL1591) cDNA was cloned into the eukaryotic expression vectors, pMT2SM. The truncated C1199 Tiam1 cDNA [carring a hemagglutinin epitope (HA)-tag at the 3' end] was cloned in the eukaryotic expression vector, pUTSV1 (Eurogentec, Belgium).

The deletion construct, HA-tagged C1199 Tiam1Δ717-727 (deleting the sequence between aa717 and aa727 of Tiam1) was derived from C1199 Tiam1 using QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). Briefly, two complimentary mutagenic oligonucleotide primers containing the desired deletion (5'CCCAACCATCAACCAGGTGTTTGAGGGAATATTTGATG-3') was designed and synthesized. First, the cycling reaction utilizing 30ng double-stranded DNA template of C1199 Tiam1 plasmid and two complimentary primers was performed to produce mutated cDNA according to the manufacturer's instruction. Subsequently, 1 ul of the Dpn I restriction enzyme (10U/ul) was added directly to the cycling reaction products in order to digest

the parental supercoiled double-stranded DNA. This Dpn I -treated cDNA was used to transform supercompetent cells (e.g. Epicurian Coli XL 1-Blue). Finally the deletion construct was confirmed by DNA sequencing.

The Tiam1 (aa393-aa728) fragment was cloned into Calmodulin-Binding-Peptide (CBP)-tagged vector (pCAL-n)(Stratagen) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, the Tiam1 fragment was amplified by PCR with two specific primers (left, 5'-AACTCGAGATGAGTACCACCAACAGTGAG-3' and right, 5'-AAAAAGCTTTCAGCCATCTGGAACAGTGTCATC-3') linked with specific enzyme digestion site (XhoI or Hind III). PCR product digested with Xho I and Hind III was purified with QIAquick PCR Purification Kit(Qiagen). The Tiam1 fragment cDNA was cloned into pCAL-n vector digested with Xho I and Hind III. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed to BL21-DE3 to produce CBP-tagged Tiam1 fragment fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma).

The Tiam1fragment cDNA was also cloned into pEGFPN1 vector (Clontech) digested with Xho I and Hind III to create GFP-tagged Tiam1 fragment cDNA. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. This GFP-tagged Tiam1 fragment cDNA was then used for transient expression in SP1 cells as described below. The GFP tagged Tiam1 fragment is expressed as a 68kDa polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses.

Cell Transfection: To establish a transient expression system, cells (e.g. SP-1 cells or COS-7 cells) were transfected with various plasmid DNAs including TimalcDNAs [e.g. the full-length mouse Tiam1cDNA (FL1591) or HA-tagged C1199 Tiam1cDNA or HA-tagged C1199 Tiam1Δ717-727 cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199 Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA (co-transfection) or vector control constructs] using electroporation methods. Briefly, cells (e.g. SP-1 cells or COS-7 cells) were plated at a density of 1 x 10^6 cells per 100 mm dish and were transfected with 25 μg/dish plasmid DNA using electroporation at 230v and 960 μFD with a Gene Pulser (Bio-Rad). Transfected cells were grown in 5% or 20% fetal calf serum-containing culture medium for at least 24-48h. Various transfectants were then analyzed for the expression of Tiam1 or HA-tagged (or GFP-tagged) Tiam1 mutant proteins by immunoblot, immunoprecipitation and functional assays as described below.

Immunoprecipitation and Immunobloting Techniques: SP-1 cells or COS cells [e.g. untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1cDNA (FL1591) or HA-tagged C1199 Tiam1cDNA or or HA-tagged C1199 Tiam1Δ717-727 cDNA were first extracted with a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Nonidet P-40 (NP-40) buffer followed by solubilizing in SDS sample buffer and analyzed by SDS-PAGE (with 7.5% gel). Separated polypeptides were then transferred onto nitrocellulose filters. After blocking non-specific sites with 3% bovine serum albumin, the nitrocellulose filters were incubated with rabbit anti-Tiam1 (5μg/ml) or mouse anti-HA ((5μg/ml) [or preimmune serum (5μg/ml)] plus peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:10,000 dilution), respectively. In controls, peroxidase-conjugated normal mouse IgG or preimmune rabbit IgG was also incubated with anti-Tiam1-mediated immuno-complex. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science, England) according to the manufacturer's instructions.

In some cases, SP-1 cells [transfected with HA-tagged C1199 Tiam1cDNAor GFP-tagged Tiam1 fragment cDNA or co-transfected with HA-tagged C1199 Tiam1cDNA and GFP-tagged Tiam1 fragment cDNA] were immunoblotted with anti-HA antibody (5μg/ml) and anti-GFP antibody (5μg/ml), respectively followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h.

In some experiments, SP-1 cells or COS cells [e.g. untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1cDNA (FL1591) or HA-tagged C1199 Tiam1cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199 Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA (co-transfection)] were immunoprecipitated with rabbit anti-Tiam1 (5μg/ml) or anti-ankyrin antibodies [e.g. rabbit anti-ANK3 antibody (5μg/ml) or mouse anti-ANK1 antibody (5μg/ml)] followed by immunobloting/rebloting with ankyrin antibodies [e.g. rabbit anti-ANK3 antibody (1μg/ml) or mouse anti-ANK1 antibody (5μg/ml)] or rabbit anti-Tiam1 (1μg/ml), respectively followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

Effects of Synthetic Peptides on Ankyrin-Tiam1 Interaction : Nitrocellulose discs (1 cm diameter) were coated with ≈ 1 μg of a panel of synthetic peptides including the ankyrin-binding region peptide (^{717}GEGTDAVKRS^{727}L), a scrambled peptide (GRATLEGSDKV) and another Tiam1-related peptide (^{399}GTIKRAPFLG^{409}P) (synthesized by Dr. Eric Smith, University of Miami). Following coating, the unoccupied sites on the discs were blocked by incubation with a solution containing 20 mM Tris-HCl (pH 7.4) and 0.3% bovine serum albumin at 4°C for 2 h. The discs were then incubated with various concentration of ^{125}I-labeled cytoskeletal proteins (erythrocyte ankyrin/ARD/ankyrin's SBD/spectrin) (≈ 3000 cpm/ng) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 % bovine serum albumin).

In some experiments, ^{125}I-labeled Tiam1 (≈ 3000 cpm/ng) was incubated with ankyrin-coated beads in the presence of various concentrations (10^{-10}M-10^{-6}M) of unlabeled synthetic peptide (e.g. ^{717}GEGTDAVKRS^{727}L or the scrambled sequence, GRATLEGSDKV or another Tiam1-related peptide, ^{399}GTIKRAPFLG^{409}P) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.2 % bovine serum albumin). In some experiments, ^{125}I-labeled Tiam1 fragment (≈ 3000 cpm/ng) was also incubated with beads containing various cytoskeletal proteins such as intact ankyrin, ARD or spectrin binding domain of ankyrin (GFP-SBD) or GFP alone. Following binding, the peptide-coated discs (or cytoskeletal protein-conjugated beads) were washed three times in the binding buffer and the radioactivity associated with peptide-coated discs (or cytoskeletal protein-conjugated beads) was estimated. As a control, the ligands were also incubated with uncoated nitrocellulose discs (or beads) to determine the binding observed due to the "stickyness" of various ligands. Nonspecific binding was observed in these controls. In the peptide competition assay, the specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

Binding of Ankyrin or ARD To Tiam1 *In Vitro*: Aliquots (0.5-1.0 μg protein) of purified Tiam1 [e.g. intact Tiam1 or C1199 Tiam1 or Tiam1 fragment] conjugated beads were incubated in 0.5 ml of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100] containing various concentrations (10-800 ng/ml) of ^{125}I-labeled intact ankyrin (purified from human erythrocytes) (5,000 cpm/ng protein) or ^{125}I-labeled recombinant ARD

fragment at 4°C for 4 h. Specifically, equilibrium binding conditions were determined by performing a time course (1-10h) of ^{125}I-labeled ankyrin (or ARD) binding to Tiam1 at 4°C. The binding equilibrium was found to be established when the in vitro ankyrin (or ARD)-Tiam1 binding assay was conducted at 4°C after 4 h. Following binding, beads were washed extensively in binding buffer and the beads-bound radioactivity was counted.

As a control, ^{125}I-labeled ankyrin or ^{125}I-labeled ARD was also incubated with uncoated beads to determine the binding observed due to the non-specific binding of various ligands. Non-specific binding which represented approximately 20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. The values expressed in the result section represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

In some cases, ^{125}I-ankyrin (1-10ng) was incubated with a polyacrylamide gel containing purified Tiam1 (obtained from anti-Tiam1 affinity column chromatography) in the absence or the presence of 100-fold excess amount of unlabeled ankyrin/spectrin (in the same binding buffer as described above) for 1h at room temperature. Following incubation, the gel was washed five times with the same binding solution and analyzed by autoradiographic analyses.

An in vitro binding assay designed to measure the stoichiometry of GST-ARD fusion protein and C1199Tiam1 was also carried out. Specifically, in each reaction, 15-60 μl of glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in 0.5 ml of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100]. Purified C1199 Tiam1 (0.5μg-1.0μg) was then added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100μg) at 4°C for 4 h. Following binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150μl of 50mM Tris-HCl (pH 8.0) buffer containing 30mM glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was then determined by SDS-PAGE and Coomassie blue staining followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199Tiam1 (mol) was then calculated. Values represent relative binding abilities averaged from three experiments ±SEM.

Binding Of ^{125}I-Labeled Ankyrin To Tiam1 and Tiam1 Mutant Proteins: Both purified wild type (the full length Tiam1) and mutant proteins (e.g. HA-tagged C1199 Tiam1 or HA-tagged C1199 Tiam1Δ717-727) were bound to the anti-Tiam1 or anti-HA immunoaffinity beads, respectively. CBP-tagged Tiam1 fragment was also bound to Sepharose beads conjugated with calmodulin. Subsequently, aliquots (10-20 ng proteins) of these beads were incubated with 0.5 ml of a binding buffer (20 mM Tris.HCl pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100) in presence of various concentrations (10-400 ng/ml) of ^{125}I-labeled ankyrin (5,000 cpm/ng protein) at 4°C for 5 h. Following binding, beads were washed extensively in binding buffer and the bead-bound radioactivity was counted.

As a control, ^{125}I-labeled ankyrin was also incubated with uncoated beads or preimmune IgG-coated beads to determine the binding observed due to the non-specific binding of the ligand. Non-specific binding which represented approximately 15-20% of the total binding, was always subtracted from the total binding. The values expressed in the result section represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

Tiam1-Mediated GDP/GTP Exchange For RhoGTPases: Purified E. coli-derived GST-tagged GTPases (e.g. Rac1, Cdc42 or RhoA) (20pmole) were preloaded with GDP (30μM) in 10μl buffer containing 25mM Tris-HCl (pH 8.0), 1mM DTT, 4.7mM EDTA, 0.16mM MgCl2 and 200μg/ml BSA at 37° for 7min. In order to terminate preloading procedures, additional MgCl2 was then added to the solution (reaching a final concentration of 9.16mM) as described previously (Zhang, et. al., 1995). Tiam1 was then isolated from COS-7 cells (transfected with either the full-lengh Tiam1cDNA or HA-tagged C1199Tiam1cDNA) or SP1 cells [transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1cDNA, GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA (as co-transfection) or vector alone] using anti-Tiam1 (or anti-HA or anti-GFP)-conjugated beads. In some cases, ankyrin-associated Tiam1 was isolated from SP1 cells [transfected with HA-tagged C1199 Tiam1cDNA, GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA (as co-transfection) or vector alone] using anti-ankyrin-conjugated beads.

Subsequently, 2pmole of Tiam1 [isolated from untransfected or transfected cells according to the procedures described above] was preincubated with no ankyrin or ankyrin [e.g. intact ankyrin or ARD (1μg/ml)] followed by adding to the reaction buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 10mM $MgCl_2$, 100uM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 2.5μM GTPγ^{35}S (≈1,250Ci/mmol). Subsequently, 2.5pmole GDP-loaded GST-tagged RhoGTPases (e.g. Rac1, Rac1 or Cdc42) or GDP-treated GST were mixed with the reaction buffer containing Tiam1 and GTPγ^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, and 10 mM $MgCl_2$ as described previously (Michiels et al., 1995; Zhang, et al., 1995). The termination reactions were then filtered immediately through nitrocellulose filters, followed by one wash with the termination buffer. The filters were dissolved completely in scintillation fluid, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTPγ^{35}S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of RhoGTPases (e.g. Rac1, Cdc42 or RhoA) was subtracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

Double Immunofluorescence Staining: SP1 cells [untransfected or transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1cDNA, GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA (as co-transfection) or vector alone] were first washed with PBS [0.1M phosphate buffer (pH 7.5) and 150mM NaCl] buffer and fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment followed by staining with different immuno-reagents. Specifically, untransfected cells were incubated with Rhodamine (Rh)-conjugated rabbit anti-ANK3 (50μg/ml) and fluorescein (FITC)-conjugated anti-Tiam1 (50μg/ml), respectively. HA-tagged C1199 cDNA transfected cells were stained with Rh-conjugated rabbit anti-ANK3 antibody (50μg/ml) and FITC-conjugated mouse anti-HA IgG (50μg/ml), respectively. GFP-tagged Tiam1 fragment cDNA transfected cells were labeled with Rh-conjugated anti-ANK3 (50μg/ml). Some SP1 transfectants [co-transfected with Tiam1 fragment cDNA and HA-tagged C1199Tiam1cDNA] were stained with Rh-conjugated anti-HA (50μg/ml) or Rh-conjugated anti-ANK3 (50μg/ml), respectively. To detect non-specific antibody binding, vector-transfected cells were labeled with Rh-conjugated anti-ANK3 (50μg/ml) followed by incubating with FITC-conjugated anti-HA (50μg/ml). No anti-HA labeling was observed in such control samples. In some experiments, GFP-tagged Tiam1 fragment cDNA transfected cells were also incubated with

Rh-labeled rabbit preimmune IgG (50μg/ml). No non-specific rhodamine staining was detected in these samples. The fluorescein- and rhodamine-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 Inverted CLSM system, Molecular Dynamics, Sunnyvale, CA).

Tumor Cell Migration and Invasion Assays: Twenty-four transwell units were used for monitoring in vitro cell migration and invasion as described previously (Bourguignon et al., 1998b; Bourguignon, et al., 2000; Merzak et al., 1994). Specifically, the 5μm porosity polycarbonate filters coated with the reconstituted basement membrane substance Matrigel (Collaborative Research, Lexington, MA) were used for the cell invasion assay (Bourguignon et al., 1998b; Merzak et al., 1994). The 5μm porosity polycarbonate filters (without Matrigel coating) were used for the cell migration assay (Bourguignon, et al., 2000; Bourguignon et al., 1998b; Merzak et al., 1994). SP-1 cells transfected with various Tiam1-related cDNAs [e.g. full-length Tiam1 cDNA or HA-tagged C1199Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (co-transfection) or vector alone] [$\approx 1 \times 10^{-4}$ cells/well in phosphate buffered saline (PBS), pH 7.2] [untreated or treated with cytochalasin D (20μg/ml) or DMSO alone] were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented by 10% fetal bovine serum were placed in the lower chamber of the transwell unit. After 18h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration and invasion processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (Zhu and Bourguignon, 2000; Merzak et al., 1994). Each assay was set up in triplicate and repeated at least 5 times. All data was analyzed statistically by Student's t test and statistical significance was set at $p<0.01$.

RESULTS

(A) Identification of The Guanine Nucleotide Exchange Factor, Tiam1 In Breast Tumor Cells (e.g. SP-1 Cells)

RhoGTPases such as Rac1 become activated when bound GDP is exchanged for GTP by a process catalysed by guanine nucleotide (GDP/GTP) exchange factors (GEFs) such as Tiam1 (T-lymphoma invasion and metastasis 1) (Habets et al., 1994). A Tiam1 transcript has previously been detected in breast cancer cells (Habets et al., 1995). In this study we have analyzed Tiam1 expression (at the protein level) in SP-1 breast tumor cells. Immunoblot analysis, utilizing anti-Tiam1 antibody designed to recognize the specific epitope located at the C-terminus of Tiam1 molecule, reveals a single polypeptide (M. W. ≈ 200kDa) (Fig. 1, lane 1). This 200kDa Tiam1-like molecule expressed in SP-1 cells is very similar to the Tiam1 detected in COS-7 cells which were transiently transfected with the full-length Tiam1 cDNA (Fig. 1, lane 2) or N-terminally truncated C1199 Tiam1cDNA [Fig. 1, lane 3-revealing primarily C1199Taim1 (160kDa) and a low level of endogeneous Tiam1 (200kDa)]. We believe that Tiam1 detected in SP-1 cells or COS-7 transfectants revealed by anti-Tiam1-mediated immunoblot is specific since no protein is detected in these cells using preimmune rabbit IgG (Fig. 1, lane 4-6).

To confirm that the Tiam1-like molecule functions as a GDP/GTP exchange factor [or a GDP-dissociation stimulator (GDS) protein] for Rac1, we have isolated Tiam1 from SP-1 cells using anti-Tiam1-conjugated Sepharose beads. Our results indicate that SP1's Tiam1 activates GDP/GTP exchange on GST-Rac1 (Fig. 2A-a) and, to a lesser extent, on GST-Cdc42 (Fig. 2A-b)

and GST-RhoA (Fig. 2A-c). The initial onset of the exchange reaction on GST-Rac1 occurs within ½-1 min after the addition of Tiam1 and the reaction reaches its maximal level approximately 16 min after Tiam1 addition (Fig. 2A-a). In contrast, the initial rate of Tiam1-catalyzed GDP/GTP exchange on Cdc42 (Fig. 2A-b) and RhoA (Fig. 2A-c) appears to be significantly lower than that detected on Rac1 (Fig. 2A-a). In control samples, the amount of [^{35}S]GTPγS associated with GST alone is found to be significantly decreased (Fig. 2A-d). Further analysis indicates that the ability of Tiam1 isolated from SP-1 cells to promote GDP/GTP exchange on Rac1 (Fig. 2B-a) is identical to that carried out by Tiam1 isolated from COS-7 transfected with the full-length Tiam1 cDNA (Fig. 2B-b) or N-terminally truncated C1199 Tiam1 cDNA (Fig. 2B-c). Therefore, we believe that Tiam1 in SP-1 cells clearly functions as a GDP/GTP exchange factor for Rho-like GTPases such as Rac1GTPase.

We have also noticed that Tiam1 isolated from non-transfected COS 7 cells grown in the presence of 20% fetal calf serum is capable of catalyzing GDP/GTP exchange on Rac1 at a much higher level (Fig. 2B-d-blank bar) than Tiam1 isolated from non-transfected COS 7 cells grown in the presence of 5% fetal calf serum (Fig. 2B-d-shaded bar). This observation is consistent with previous findings that some serum components play an important role in up-regulating the ability of Tiam1 to promote GDP/GTP exchange on Rac1 (Stam et al., 1998). In SP1 cells (Fig. 2B-a-blank and shaded bars) or Tiam1cDNA-transfected COS7 cells (Fig. 2B-b-blank and shaded bars; 2 B-c-blank and shaded bar), neither high nor low serum causes significant changes in the ability of Tiam1 to catalyze GDP/GTP exchange on Rac1. These differential serum effects on the activity of Tiam1 isolated from low or high Tiam1 expressing cells awaits future investigation.

(B) <u>Interaction of Tiam 1 and the Cytoskeletal Proteins, Ankyrin</u>

Certain cytoskeleton proteins, such as ankyrin, are known to be involved in regulating a variety of cellular activities (Bennett, 1992; Bennett and Gilligan, 1993; De Matteis and Morrow, 1998; Bourguignon, 1996; Bourguignon, et al., 1998a). Both ankyrin1 (ANK1) and ankyrin3 (ANK3) have been shown to be expressed in breast tumor cells (Bourguignon, et al., 1998b; Bourguignon, et al. 1999). In this study we have carried out anti-ANK1 or anti-ANK3-mediated immunoprecipitation of SP-1 cellular proteins followed by anti-Tiam1 immunoblot (Fig. 3A, lane 2; Fig. 3B, lane 2) and anti-ANK1 (Fig. 3A-lane 3)/ANK3 (Fig. 3B-lane 3) immunoblot, respectively. Our results indicate that the Tiam1 band is revealed in anti-ankyrin (e.g. ANK1 or ANK3)-mediated immunoprecipitated materials (Fig. 3A, lane 2; Fig. 3B, lane 2). Apparently, Tiam1 is co-precipitated with ANK1 and/or ANK3 (revealed by reblotting with anti-ANK1/ANK3 antibody) (Fig. 3A, lane 3; Fig. 3B, lane 3). In control samples, immunobloting of anti-ANK1 or anti-ANK3-immunoprecipitated material using rabbit preimmune serum (Fig. 3A, lane 1; Fig. 3B, lane 1) does not reveal any protein associated with this material. Anti-Tiam1-mediated immunoprecipitation of SP-1 cellular proteins followed by anti-ANK1 (Fig. 3A, lane 4) or anti-ANK3 (Fig. 3B, lane 4)-mediated immunoblot also shows that both ANK1 (Fig. 3A, lane 4) and ANK3 (Fig. 3B, lane 4) can be co-precipitated with Tiam1 (revealed by reblotting with anti-Tiam1 antibody) (Fig. 3A, lane 5; Fig. 3B, lane 5). In controls, very little material is detected in this anti-Tiam1-mediated immuno-complex using either normal mouse IgG (Fig. 3A, lane 6; Fig. 3B, lane 6) or rabbit preimmune serum-mediated immunoblot (Fig. 3A, lane 7; Fig. 3B, lane 7). These findings clearly establish the fact that Tiam1 and ankyrin (e.g. ANK1 and ANK3) are closely associated with each other as a complex <u>in vivo</u> in breast tumor cells.

Further analyses utilizing an <u>in vitro</u> binding assay show that ^{125}I-labeled ankyrin [i.e. erythrocyte ankyrin (ANK1)] binds Tiam1 (isolated from SP1 cells) specifically (Fig. 4A-a). In addition, we have used ^{125}I-ankyrin to bind purified Tiam1 (isolated from SP-1 cells) on a gel (Fig.

4B-a). Our data indicate that Tiam1 binds to ankyrin (ANK1) (Fig. 4B-a) directly. In the presence of an excess amount of unlabeled ankyrin, the binding between ankyrin and Tiam1 is greatly reduced (Fig. 4A-b and 4B-b). Other cytoskeletal proteins, such as spectrin, do not interfere with ankyrin binding to Tiam1 (Fig. 4A-c and 4B-c). However, the precise functional domain(s) of ankyrin involved in Tiam1 binding remains to be determined.

The N-terminal region of ankyrin's membrane binding domain (Fig. 5A-a) is comprised of a tandem array of 24 ankyrin repeats (so-called ankyrin repeat domain, ARD) (Fig. 5A-b). The question of whether the membrane-binding domain of ankyrin (in particular, the ankyrin repeat domain, ARD) is involved in Tiam1 binding is now addressed in this study. First, the pGEX-2TK recombinant plasmid encoding ARD (N-terminal portion of ankyrin, from aa 1 to 834) was constructed with a GST tag and expressed in E. coli (Zhu and Bourguignon, 2000). The purified GST-tagged ARD fusion protein is expressed as a 116 kDa protein (Fig. 5B-lane 1). After the removal of GST tag by thrombin digestion, the ARD itself is found to be a 89 kDa polypeptide (Fig. 5B, lane 2), which is similar to the 89 kDa ankyrin repeat domain (ARD) obtained by enzymatic digestion of erythrocyte ankyrin (Davis and Bennett, 1990).

Next, we have used the ARD fragment of ANK3 (GST-ARD) and purified Tiam1 to identify the exact Tiam1 binding site(s) on the ankyrin molecule. Specifically, we have tested the binding of Tiam1 to ^{125}I-labeled intact erythrocyte ankyrin (ANK1) [or ^{125}I-labeled GST-ARD fragment of ANK3] under equilibrium binding conditions. Scatchard plot analyses indicate that intact erythrocyte ankyrin (ANK1) binds to Tiam1 at a single site (Fig. 5C) with high affinity [an apparent dissociation constant (K_d) of $\approx$ 0.72 nM]. This ankyrin-Tiam1 binding interaction is comparable in affinity to Tiam1 binding (Kd $\approx$ 1.42 nM) to ANK3's ARD fragment (Fig. 5D). These findings strongly support the notion that ankyrin [in particular, the ankyrin repeat domain (ARD)] is involved in Tiam1 binding site.

(C) Determination of Tiam1's Ankyrin-Binding Domain

Previous studies indicate that Tiam1's NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (i.e. a sequence between aa393-aa738 of Tiam1) (Fig. 6A-a,b,c) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and C-Jun NH2-terminal kinase activation (Michiels, et al., 1997; Stam, et al., 1997). Using a 49kDa E. coli-derived calmodulin-binding peptide (CBP)-tagged Tiam1 fragment (i.e. aa393-aa738 of Tiam1) (Fig. 6B, lane 1) and an in vitro binding assay (Fig. 6C), we have detected a specific binding interaction between the Tiam1 fragment and ankyrin (Fig. 6C-a) and ARD (Fig. 6C-b) but not the spectrin binding domain of ankyrin (Fig. 6C-c) or spectrin (Fig. 6C-d).

Furthermore, we have evaluated the binding interaction between GST-ARD fusion protein and the recombinant C1199 Tiam1 (N-terminally truncated Tiam1) (Fig. 6D). First, glutathione-Sepharose beads containing GST-ARD were incubated with C1199 Tiam1 in the absence (Fig. 6D, lane 1; and Fig. 6E-lane 1) or the presence of an excess amount of Tiam1 fragment (Fig. 6D, lane 2; and Fig. 6E, lane 2). In controls, C1199 Tiam1 was also added to Sepharose beads containing GST alone (Fig. 6D, lane 3; and Fig. 6E, lane 3). Following binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using a buffer containing glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was then determined by SDS-PAGE and Coomassie blue staining (Fig. 6E) followed by densitometric scanning analyses (Fig. 6D). Our results indicate that the stoichiometry of ARD:C1199 Tiam1 interaction is approximately 1:1 (Fig. 6D, lane 1; Fig. 6E, lane 1a and b). In the presence of an excess amount ($\approx$100-fold) of recombinant Tiam1 fragment, the binding between ankyrin ARD and C1199Tiam1 is significantly reduced (Fig. 6D-lane 2a and b; and

Fig. 6E-lane 2a and b). The control beads containing GST alone fail to bind C1199 Tiam1 (Fig. 6D, lane 3; and Fig. 6E, lane 3a and b). These observations suggest that (i) ankyrin ARD directly interacts with Tiam1; and (ii) the ankyrin-binding domain (ARD)-containing Tiam1 fragment act as a potent competitive inhibitor of Tiam1 binding to ankyrin in vitro.

Protein sequence analyses show that Tiam1 contains the sequence "^{717}GEGTDAVKRS^{727}L" (in mouse), or "^{717}GEGTEAVKRS^{727}L (in human) which shares a great deal of sequence homology with the ankyrin binding domain of the cell adhesion receptor, CD44 family (Lokeshwar, et al., 1994; Zhu and Bourguignon, 1998). To test whether the sequence "GEGTDAVKRSL" of Tiam 1 protein is in fact involved in ankyrin binding, we have examined the ability of an 11 amino acid synthetic peptide, identical to "GEGTDAVKRSL", to bind various cytoskeletal proteins. As shown in Table 1, this synthetic peptide binds specifically to intact ankyrin and the ankyrin-repeat domain (ARD), but not ankyrin's spectrin binding domain (SBD) or other cytoskeletal proteins such as spectrin. Control peptides, containing the scrambled sequence (GRATLEGSDKV) with the same amino acid composition as that of the synthetic peptide or another peptide (GTIKRAPFLGP) from different region (i.e. the sequence between aa399 and aa409) of Tiam1, fail to bind any cytoskeletal proteins tested (Table 1).

We have also used the synthetic peptide corresponding to Tiam1's aa717-aa727 sequence to compete for the binding of purified Tiam1 to ankyrin. As shown in Fig. 7A, the synthetic peptide competes effectively with Tiam1 to bind ankyrin with an apparent inhibition constant (K_i) ≈ 0.5 nM (Fig. 7A-c). However, control peptides such as GRATLEGSDKV (Fig. 7A-a) or GTIKRAPFLGP (Fig. 7A-b) do not compete at all with Tiam1 in ankyrin binding. These result suggest that the aa717-aa727 sequence of Tiam1 is a critical part of the ankyrin binding domain of Tiam1. Finally, we have constructed an HA-tagged C1199 Tiam1 deletion mutant lacking the ankyrin binding sequence, aa717-aa727 (designated as C1199 Tiam1Δ717-727) (Fig. 7B-b). The truncated C1199 Tiam1Δ717-727 cDNA (Fig. 7B-b)and the wild-type C1199 Tiam1(Fig. 7B-a) were then transiently transfected into SP-1 cells, respectively. Our results indicate that both the C1199 Tiam1Δ717-727 mutant (Fig. 7C, lane 3) and the wild-type C1199 Tiam1 (Fig. 7C-lane 2) are expressed as a 160 kDa polypeptide in SP-1 transfectants using anti-HA-mediated immunoblotting. No protein band was detected in vector-transfected SP-1 cells (Fig. 7C, lane 1). Further analyses using anti-HA-conjugated immuno-beads indicate that the HA-tagged C1199 Tiam1Δ717-727 mutant protein isolated from SP-1 transfectants displays a drastic reduction (approximately ≈90-95% inhibition) in ankyrin-binding ability (Fig. 7D-c) compared to the HA-tagged wild-type C1199 Tiam1 (Fig. 7D-b). No ankyrin binding is observed in materials associated with anti-HA-beads isolated from vector-transfected cells (Fig. 7D-a). These finding suggest that the aa717-aa727 region is critical for the interaction of Tiam1 with ankyrin.

Most importantly, we have found that the binding of ankyrin [e.g. erythrocyte ankyrin (ANK1) (Fig. 8A) or ANK3's ARD (Fig. 8B)] to Tiam1 significantly increases the GDP/GTP exchange activity of Rac1 GTPase as compared to untreated Tiam1-mediated Rac1 activation (Fig. 8C). The spectrin binding domain of ankyrin or other cytoskeletal proteins, such as spectrin, fail to stimulate Tiam1-mediated GDP/GTP exchange on Rac1 GTPase (data not shown). Therefore, we believe that ankyrin binding to Tiam 1 plays a pivotal role in the up-regulation of Tiam 1-mediated GDP-GTP exchange activity of Rho-like GTPases (e.g. Rac1).

(D) Effect of Tiam1 or the Tiam1 Fragment On Rac1 Activation, Tumor Cell Invasion and Migration

Previous studies have indicated that both ankyrin and Tiam1 are closely associated with certain tumor-specific behaviors, characterized by an "invadopodia" structure (or membranous projections) (Mueller and Chen, 1991; Monsky, et al., 1994) and epithelial tumor cell migration (Bourguignon et al., 1998a; 1998b; Zhu and Bourguignon, 2000; Bourguignon, et al., 2000). In this study using double immuno-labeling staining, we have observed that both ankyrin (Fig. 10A) and Tiam1 (Fig. 10B) are co-localized in the plasma membrane and long projections of SP1 cells (Fig. 10C). Furthermore, we have transiently transfected breast tumor cells (e.g. SP-1 cells) with HA-tagged N-terminally truncated C1199 Tiam1 cDNA. Our results show that the C1199 Tiam1 is expressed as a 160 kDa protein (Fig. 9B-a) detected by anti-HA-mediated immunoblot in SP1 cells. No protein band was detected in vector-transfected SP1 cells by anti-HA-mediated immunobloting (Fig. 9A-a). Double immunofluorescence staining data show that ankyrin (Fig. 10D) and C1199Tiam1 (Fig. 10E) are also co-localized on the plasma membrane-related long projections of these C1199cDNA-transfected cells (Fig. 10F). Furthermore, we have demonstrated that transfection of SP1 cells with C1199 Tiam1 cDNA stimulates ankyrin-associated Tiam1-catalyzed GDP/GTP exchange on Rac1 (Fig. 11a), and induces a significant amount of increase in breast tumor cell invasion (Table 2A) and migration (Table 2B) as compared to vector-transfected SP1 transfectants (11b and Table 2A,B). These results are consistent with previous findings indicating that transfection of NIH3T3 cells with the N-terminally truncated C1199 Tiam1 cDNA confers potent oncogenic properties (Van Leeuwen, et al., 1995).

Treatment of SP1 cells (e.g. untransfected cells or transfected cells) with certain agents [e.g. cytochalasin D (a microfilament inhibitor)] causes a remarkable inhibition of tumor cell invasion (Table 2A) and migration (Table 2B). Tiam1-Rac1 signaling initiates oncogenic cascades including c-Jun Kinase (JNK) activation which triggers gene transcription through c-jun and promotes cell transformation (Michiels, et al., 1995; Michiels, et al., 1997). In addition, Tiam1-activated Rac1 stimulates the novel family of serine/threonine kinases, Paks (p-21 activated kinases) (Manser, et al.,1994; Knaus, et al., 1995; Bagrodia and Cerione, 1999) which mediate actin assembly and induce the formation of membrane ruffling and lamellipodia (membrane projections). In fact, cytoskeleton-associated membrane projections are often tightly linked to matrix degradating enzymes during breast tumor cell invasion and migration (Bourguignon, et al. 1998b). These findings suggest that Tiam1-Rac1 signaling and selective effector(s) play an important role in promoting certain gene expression required for cellular transformation and the up-regulation of cytoskeletal changes needed for tumor cell invasion and migration. Identification of immediate downstream targets for ankyrin-mediated Tiam1-Rac1 signaling is currently under investigation in our laboratory.

We have also found that SP1 cells transfected with GFP-tagged Tiam1 fragment cDNA express a 68 kDa protein as detected by anti-GFP antibody (Fig. 6B, lane 2; Fig. 9C-b). In vector-transfected SP1 cells, we are not able to detect any protein band by anti-GFP-mediated immunobloting (Fig. 9A-b). Double immunofluorescence staining shows that both ankyrin (Fig. 10G) and the GFP-tagged Tiam1 fragment (Fig. 10H) are co-localized in the cell membranes in SP1 transfectants (Fig. 10I). We believe that ankyrin staining detected in these SP1 transfectants revealed by anti-ankyrin-mediated immuno-staining is specific since no label (Fig. 10a) is detected in these GFP-Tiam1 fragment overexpressed cells (Fig. 10b) using preimmune rabbit IgG (Fig.10a). No co-localization (Fig. 10c) of preimmune IgG (Fig. 10a) and GFP-Tiam1 fragment (Fig. 10b) is observed in these transfectants. Moreover, we have demonstrated that overexpression of GFP-tagged Tiam1

fragment in SP1 transfectants downregulates ankyrin-associated Tiam1-Rac1 signaling (Fig. 10d) and tumor cell invasion (Table 2A) as well as cell migration (Table 2B).

Finally, co-transfection of SP1 cells with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA was carried out. Using anti-HA or anti GFP-mediated immunoblotting technique, we have detected co-expression of both C1199 Tiam1 (Fig. 9D-a) and Tiam1 fragment (Fig. 9D-b) in SP1 transfectants. In control experiments, no detectable signal was observed when these transfectants were immunoblotted with anti-GFP (Fig. 9B-b) or anti-HA (Fig. 9C-a), respectively. Furthermore, immunocytochemical staining results show that ankyrin (Fig. 10d) and the GFP-tagged Tiam1 fragment (Fig. 10e) are co-localized (Fig. 10f) in the plasma membranes of SP1 transfectants. In contrast, C1199 Tiam1 (Fig. 10J) fails to display plasma membrane localization. Consequently, the amount of co-localization (Fig. 10L) between C1199 Tiam1 (Fig. 10J) and Tiam1 fragment (Fig. 10K) is greatly reduced. In addition, it is noted that no significant stimulation of long membrane projections was observed in these transfectants (Fig. 10 J,K,L; and 10 d,e,f). Other tumor-specific behaviors such as Tiam1-Rac1 activation (Fig. 11c) and cytoskeleton-mediated breast tumor cell invasion (Table 2A) and migration (Table 2B) are also greatly inhibited. These findings suggest that the ankyrin-binding domain containing Tiam1 fragment acts as a dominant-negative mutant which effectively competes for ankyrin binding to C1199Tiam1 in vivo and blocks ankyrin-regulated Tiam1 function associated with tumor-specific phenotypes.

KEY RESEARCH ACCOMPLISHMENTS:

•We have found that Tiam1 (T lymphoma invasion and metastasis) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for RhoGTPases (e.g. Rac1) and is expressed in breast tumor cells (e.g. SP-1 cell line).

(A)Tiam1 Interaction With the Cytoskeletal Protein, Ankyrin:

•Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo.

•The ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding; the 11aa sequence between aa717 and aa727 of Tiam1 (^{717}GEGTDAVKRS^{727}L) is the ankyrin binding domain.

•Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on RhoGTPases (e.g. Rac1).

•Using an Escherichia coli-derived calmodulin-binding peptide (CBP)-tagged recombinant Tiam1 (aa393-aa728) fragment which contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (aa393-aa738) fragment and ankyrin in vitro. This Tiam1 fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin.

•Transfection of SP-1 cell with Tiam1 cDNAs stimulates (i)Tiam1-ankyrin association in the membrane projections, (ii) Rac1 activation and (iii) breast tumor cell invasion and migration. Co-transfection of SP1 cells with green fluorescent protein (GFP)-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1-ankyrin co-localization in the cell membrane and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes.

(B) Tiam1 Interaction With The Metastasis-Specific Molecule, CD44 [the hyaluronic acid (HA) binding receptor]:

•We have also explored the interaction between the metastasis-specific molecule, CD44 [the hyaluronic acid (HA) binding receptor] and Tiam1 (a guanine nucleotide exchange factor) in metastatic breast tumor cells (SP1 cell line).

•Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in Tiam1's PHn-CC-Ex domain with an apparent dissociation constant (Kd) of 0.2nM which is comparable to CD44 binding (Kd ≈0.13nM) to intact Tiam1. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1 binding region for CD44.

•Most importantly, the binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration.

•Transfection of SP1 cells with Tiam1cDNA promotes Tiam1 association with CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with PHn-CC-Ex cDNA and Tiam1 cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors.

•These observations clearly suggest that Tiam1 contains multiple functional domains [e.g. membrane localization site(s) and cytoskeleton binding domains] required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function leading to metastatic breast tumor cell progression.

REPORTABLE OUTCOMES:

•Manuscripts and abstracts: see section #13 (final report section).

•Funding applied for based on work supported by this award:

Funded Active Grants:

NCI Grant (2000-2005)"CD44/Variant-Cytoskeleton In Breast Cancer Progression".

NCI Grant (1999-2003) "CD44-p185^{HER2} Interaction In Ovarian Cancer Progression".

US Army Breast Cancer Grant (DOD) (1999-2002) "A Novel Signaling Perturbation and Ribozyme Gene Therapy Proceudre to Block Rho-Kinase (ROK) Activation and Breast Tumor Metastasis".

CONCLUSIONS:

The invasive phenotype of breast tumors, determined by characteristics such as tumor cell motility and membrane perturbations, is clearly linked to cytoskeletal function. For example, recent studies have shown that certain metastasis-specific molecules [e.g. $CD44v_{3,8-10}$ isoform (Bourguignon, et al., 1998b; Bourguignon, et al., 1999) and its associated matrix metalloproteinase, MMP-9 (Bourguignon, et al., 1998b; Yu and Stamenkovic, 1999), as well as Rho-Kinase (ROK) (Bourguignon, et al., 1999)] are closely associated with the cytoskeleton during tumor cell function. In order to further examine the regulatory mechanism(s) involved in cytoskeleton-mediated oncogenic signaling leading to tumor cell invasion and migration, we have focused on the guanine nucleotide exchange factors (GEFs-the Dbl or DH family) such as Tiam1 which are known to display oncogenic capability and function as upstream activators of Rho-like GTPases (e.g. Rac1 or Cdc42) (Habets et al., 1994; Michiels et al., 1995; Woods et al., 1991; van Leeuwen et al.,1995; Nobes and Hall, 1995). In breast tumor cells, such as SP-1 cells, Tiam1 is detected as a 200 kD protein (Fig. 1) that is similar to Tiam1 described in other cell types (Michiels et al., 1995; Woods et al., 1991; van Leeuwen et al.,1995; Nobes and Hall, 1995; Stam et al., 1997; Hordijk et al., 1997; van Leeuwen et al., 1997; Bourguignon, et al., 2000). Tiam1 isolated from SP-1 cells is also capable of carrying out GDP/GTP exchange for Rac1 in vitro (Fig. 2). Sequence analysis of Tiam1 suggests that its association with the invasive and metastatic phenotype is mediated via membrane-linked cytoskeletal regulation and/or activation of Rho family GTPases (Habets et al., 1994; Nobes and Hall, 1995).

Rac1 acts downstream of Tiam1 signaling and regulates the function of several cell adhesion molecules such as the laminin receptor, α6β1 integrin (van Leeuwen et al., 1997), E-cadherin (Hordijk et al., 1997) and the hyaluronan receptor, CD44 (Bourguignon, et al., 2000). Tiam1-Rac1 activation has also been shown to be stimulated by certain serum-derived growth activators [e.g. sphingosine-1-phosphate (S1P) and LPA] during T-lymphoma cell invasion (Stam et al., 1998). However, in epithelial Madin-Darby canine kidney (MDCK) cells, Tiam1-Rac1 signaling plays an invasion-suppressor role in Ras-transformed MDCK cells (Hordijk et al., 1997). Apparently, various responses by Tiam1-catalyzed Rac1 signaling may be controlled by selective upstream activators [e.g. availability of certain cytoskeletal proteins (e.g. ankyrin), cell adhesion receptors (e.g. CD44, integrin or E-cadherin), growth activators (e.g. serum, S1P or LPA) or extracellular matrix components (hyaluronic acid, collagen or fibronectin, etc.]. Moreover, Tiam1 is found to be involved in promoting both Rac1- and RhoA-mediated pathways during neurite formation in nerve cells (van Leeuwen et al., 1997). The balance between Rac1 and RhoA determines a particular cellular morphology and migratory behaviors (Sander, et al., 1999).

Ankyrin is a family of membrane-associated cytoskeletal proteins expressed in a variety of biological systems including epithelial cells and tissues (Peters and Lux, 1993). Presently, at least three ankyrin genes have been identified: ankyrin 1 (Ank 1 or ankyrin R), ankyrin 2 (Ank 2 or ankyrin B) and ankyrin 3 (Ank 3 or ankyrin G) (Lux et al., 1990; Otto, et al., 1991; Kordeli, et al., 1995; Peters, et al., 1995). These molecules belong to a family of related genes that probably arose by duplication and divergence of a common ancestral gene. Ankyrin is known to bind to a number of plasma membrane-associated proteins including band 3, two other members of the anion exchange gene family (Bennet, 1992), Na^+/K^+-ATPase (Nelson and Veshnock, 1987; Zhang, et al., 1998), the amiloride-sensitive Na^+ channel (Smith et al., 1991), the voltage-dependent Na^+ channel (Kordeli et al., 1995), Ca^{2+} channels (Bourguignon et al., 1993b; Bourguignon and Jin, 1995; Bourguignon et al., 1995a) and the adhesion molecule, CD44 (Bourguignon, et al., 1986; Kalomiris and Bourguignon, 1988; Kalomiris and Bourguignon, 1989; Lokeshwar and Bourguignon, 1991; Lokeshwar and

Bourguignon, 1992; Bourguignon, et al., 1992; Bourguignon, et al., 1991; Lokeshwar, et al., 1994; Lokeshwar, et al., 1996; Bourguignon, 1996). It has been suggested that the binding of ankyrin to certain membrane-associated molecules is necessary for signal transduction, cell adhesion, membrane transport, cell growth, migration and tumor metastasis (Bennet, 1992; Bourguignon et al., 1995b; Bourguignon, 1996; Bourguignon et al., 1997; Bourguignon et al., 1998a; De Matteis and Morrow, 1998; Zhu and Bourguignon, 1998; Zhu and Bourguignon, 2000).

In this study we have presented a new evidence showing the interaction between ankyrin and Tiam1. Specifically, we have demonstrated that Tiam1 and ankyrin (e.g. ANK1 and ANK3) are physically linked to each other as a complex in vivo (Figs. 3 and 10) and in vitro (Figs. 5,6 and 7); and that ankyrin binding to Tiam1 promotes Rac1 activation (Figs. 8 and 11). Using purified Tiam1 and GST-tagged ankyrin repeat domain (GST-ARD) (Fig. 5) to examine the interaction between Tiam1 and ankyrin in vitro, we have found that the ankyrin repeat domain (ARD) is directly involved in the binding of Tiam1 (Fig. 5 C and D; and Fig. 6 C, D and E). In fact, the binding affinity of ARD to Tiam1 is very comparable to that of intact erythrocyte ankyrin binding to Tiam1 (Fig. 5C and D). These findings support the conclusion that the ARD fragment of ankyrin is directly involved in the recognition of Tiam1. The 24 ankyrin repeats within the ARD are known to form binding sites for at least seven distinct membrane protein families (Michaely and Bennett, 1995). Often, the ARD is organized into four folding subdomains [e.g. subdomain1 (S1), subdomain 2 (S2), subdomain 3 (S3) and subdomain 4 (S4)]. Recently, we have shown that the subdomain 2 (S2) (but not other subdomains) of ARD binds to the adhesion molecule, CD44 directly (Zhu and Bourguignon, 2000). Overexpression of subdomain (S2) of ARD promotes CD44-mediated tumor cell migration (Zhu and Bourguignon, 2000). The question of which ARD subdomain fragment(s) is(are) involved in regulating Tiam1 function remains to be determined.

The structural homology between the ankyrin binding domain of Tiam1 (the sequence between aa717 and aa727) and CD44 is quite striking (Lokeshwar, et al. 1994). The cytoplasmic domain of CD44 (approximately 70 a. a. long) is highly conserved (≥ 90%) in most of the CD44 isoforms; and it is clearly involved in specific ankyrin binding (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). The ankyrin-binding domain of CD44 has also been mapped using deletion mutation analyses and mammalian expression systems (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). In particular, the ankyrin-binding domain [e.g. "NGGNGTVEDRKPSEL" between aa 306 and aa320 in the mouse CD44 (Lokeshwar et al., 1994) and "NSGNGAVEDRKPSGL " aa304 and aa318 in human CD44 (Zhu and Bourguignon, 1998)] is required for cell adhesion (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998), the recruitment of Src kinase (Zhu and Bourguignon, 1998) and the onset of tumor cell transformation (Zhu and Bourguignon, 1998; Bourguignon, et al. 1998b). The facts that : (a) the recombinant C1199Tiam1 interacts with ARD fusion protein directly with a stoichiometry of 1:1 (Fig. 6D and E); (b) a peptide with the sequence ^{717}GEGTDAVKRS^{727}L of Tiam1 binds to ankyrin and ARD but not ankyrin's spectrin binding domain or spectrin (Table 1); and (c) a Tiam1 peptide (aa717-aa727) competes with Tiam1 for the binding to ankyrin (Fig. 7A); (d) the Tiam1 deletion mutant protein (e.g. C1199 Tiam1Δ717-727) (Fig. 7B) fails to bind ankyrin (Fig. 7D); and (e) ankyrin stimulates Tiam1-catalyzed GDP/GTP exchange activity on Rac1 (Fig. 8) strongly suggest that the sequence (^{717}GEGTDAVKRS^{727}L) of Tiam1 is an important region for ankyrin binding. Furthermore, we have shown that transfection of SP-1 cells with HA-tagged N-terminally truncated C1199 Tiam1 cDNA stimulates ankyrin-associated GDP/GTP exchange on Rac1 (Fig. 11) as well as tumor cell invasion (Table 2A) and migration (Table 2B). These Tiam1-activated oncogenic responses are consistent

with previous studies indicating Tiam1-activated Rho-like GTPases may act as downstream effectors of Ras in both tumorigenesis and progression to metastastic diseases (Habets et al., 1994; Habets et al., 1995; van Leeuwen et al., 1995).

The aa393-aa738 Tiam1 fragment (Fig. 6C) contains not only the putative ankyrin-binding domain (aa717-aa727) but also NH2-terminal pleckstrin homology (PHn), the coiled coil region (CC) and an additional adjacent region (Ex) [also designated as PHn-CC-Ex domain] (Michiels et al., 1997). This Tiam1 fragment has been shown to be responsible for Tiam1's membrane localization, Rac1-dependent membrane ruffling and C-Jun NH_2-terminal kinase activation in fibroblasts and COS cells (Stam et al., 1997; Michiels et al., 1997). In this study we have found that co-transfection of SP1 cells with Tiam1 fragment cDNA and C1199 Tiam1cDNA effectively blocks tumor cell-specific behaviors [e.g. C1199 Tiam1 association with ankyrin in the cell membrane (Fig. 10), Rac1 activation (Fig. 11), tumor cell invasion (Table 2A) and migration (Table 2B)]. These findings further support our conclusion that the ankyrin-binding domain-containing Tiam1 fragment acts as a potent competitive inhibitor which is capable of interfering with C1199 Tiam1-ankyrin interaction in vivo. Recently, we have also demonstrated that the Tiam1 fragment is required for CD44 (the hyaluronan receptor) binding (Bourguignon, et al., 2000). Most importantly, Tiam1-CD44 interaction promotes Rac1 activation and hyaluronic acid-mediated breast tumor cell migration (Bourguignon, et al., 2000). These observations clearly suggest that the aa393-aa738 of Tiam1 contains multiple functional domains [e.g. membrane localization site(s) and cytoskeleton binding domains] required for the regulation of Tiam1-Rac1 signaling and cytoskeleton function. Taken together, we believe that ankyrin-Tiam1 interaction plays a pivotal role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor cell progression.

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APPENDICES:

Table 1: Binding of ^{125}I-labeled cytoskeletal proteins to synthetic peptides.

A: Binding To "GEGTDAVKRSL" (The sequence between aa717 and aa727 of Tiam1):

	(nM x CPM Bound)
^{125}I-labeled Intact Ankyrin	15,260 ± 120
^{125}I-labeled ARD	14,560 ± 105
^{125}I-labeled Ankyrin's Spectrin Binding Domain	770 ± 22
^{125}I-labeled Spectrin	850 ± 34

B: Binding To "GRATLEGSDKV" (The scrambled sequence):

	(nM x CPM Bound)
^{125}I-labeled Ankyrin	1,020 ± 36
^{125}I-labeled ARD	920 ± 29
^{125}I-labeled Ankyrin's Spectrin Binding Domain	901 ± 24
^{125}I-labeled Spectrin	996 ± 27

C: Binding To "GTIKRAPFLGP: (The sequence between aa399 and aa409 of Tiam1)

	(nM x CPM Bound)
^{125}I-labeled Ankyrin	899 ± 23
^{125}I-labeled ARD	854 ± 17
^{125}I-labeled Ankyrin's Spectrin Binding Domain of	842 ± 19
^{125}I-labeled Spectrin	863 ± 20

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^{125}I-labeled cytoskeletal proteins [e.g. intact ankyrin (100ng) or ARD (100ng) or spectrin binding domain of ankyrin (100ng) or spectrin (100ng)] were incubated with nitrocellulose discs coated with either the synthetic peptide "GEGTDAVKRSL" (corresponding to the sequence between aa717 and aa727 of Tiam1) or the scrambled peptide "GRATLEGSDKV" or another Tiam1-related peptide "GTIKRAPFLGP" (corresponding to the sequence between aa399 and aa409 of Tiam1) at 4°C for 4h as described in the Materials and Methods. As a control, the radiolabelled ligands including ^{125}I-labeled ankyrin, ^{125}I-labeled ARD and ^{125}I-labeled spectrin were also incubated with uncoated beads to determine the binding observed due to the non-specific binding of various ligands. Non-specific binding which represented approximately 20% of the total binding, was always subtracted from the total binding.

Table 2: Measurement of Tumor Cell Invasion and Migration.

A: In vitro cell invasion:

Cells	Cell Invasion[a] (% of Control)[b] DMSO-treated control	Cytochalasin D-treated
Untransfected cells (control)	100	22
Vector-transfected cells	96	24
Tiam1 fragment cDNA-transfected cells	95	20
C1199 Tiam1 cDNA-transfected cells	155	50
C1199 Tiam1cDNA and Tiam1 fragment cDNA co-transfected cells	90	17

B: In vitro cell migration:

Cells	Cell Migration[a] (% of Control)[b] DMSO-treated control	Cytochalasin D-treated
Untransfected cells (control)	100	20
Vector-transfected cells	98	23
Tiam1 fragment cDNA-transfected cells	93	22
C1199 Tiam1cDNA-transfected cells	158	55
C1199 Tiam1cDNA and Tiam1 fragment cDNA co-transfected cells	88	14

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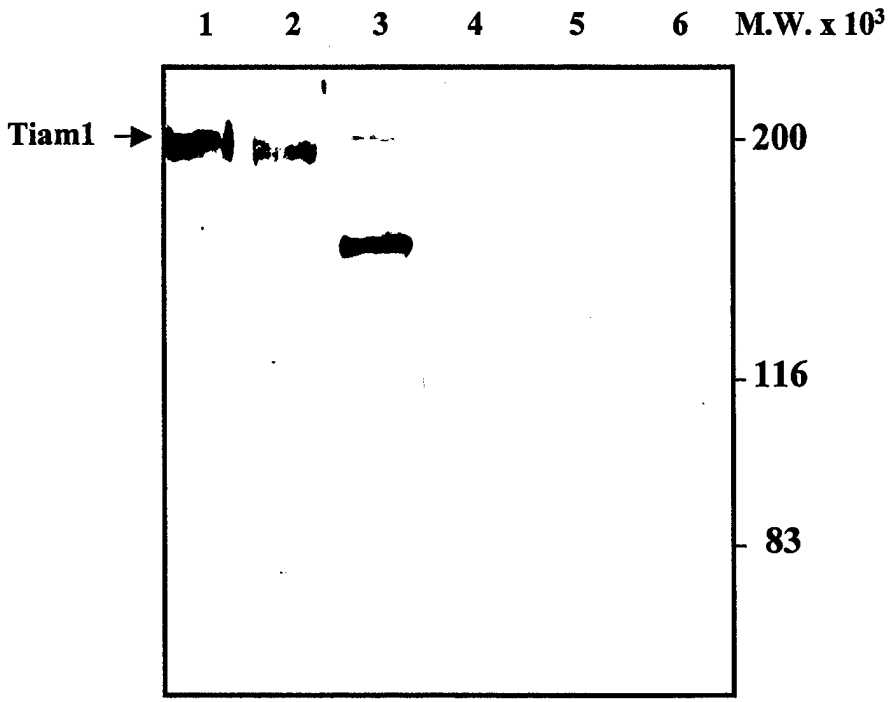
a: SP1 cells [$\approx 1 \times 10^4$ cells/well in phosphate buffered saline (PBS), pH 7.2] [in the presence or absence of 20μg/ml cytochalasin D (dissolved in DMSO) or DMSO alone] were placed in the upper chamber of the transwell unit. In some cases, SP1 cells were transfected with either HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199 Tiam1cDNA plus GFP-tagged Tiam1 fragment cDNA , or vector alone. After 18h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters containing serum by standard cell number counting assays as described in the Materials and Methods. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$. In these experiments ≈30 to 40% of input cells ($\approx 1 \times 10^4$ cells/well) undergo in vitro cell invasion and migration in the control samples.

b: The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

FIGURE 1



Detection of Tiam1 expression in SP-1 cells or COS-7 transfectants.

SP-1 cells and COS-7 cells [transfected with the full-length Tiam1cDNA (FL1591) or N-terminally truncated C1199 Tiam1 cDNA or vector alone] were solubilized in SDS sample buffer and analyzed by SDS-PAGE and immunoblot as described in the Materials and Methods.

Lane 1: Anti-Tiam1-mediated immunoblot of SP-1 cells.

Lane 2: Anti-Tiam1-mediated immunblot of COS-7 cells transfected with the full-length Tiam1cDNA (FL1591).

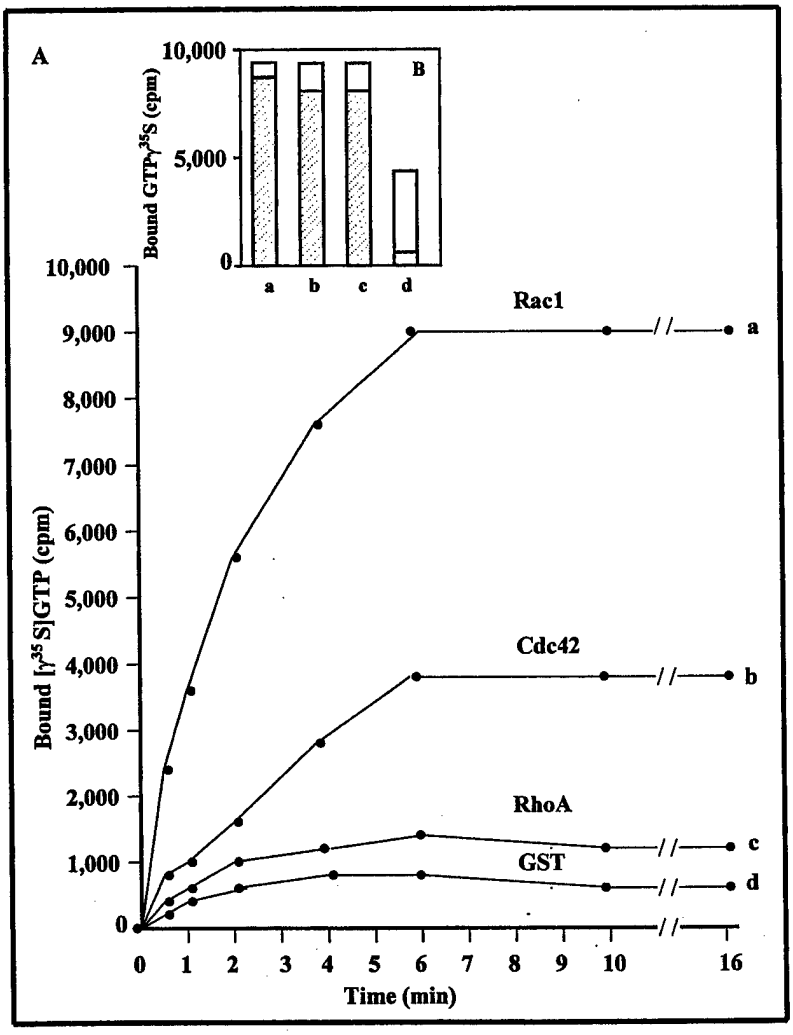
Lane 3: Anti-Tiam1-mediated immunoblot of COS-7 cells transfected with the N-terminally truncated C1199 Tiam1cDNA.

Lane 4: Immunoblot of SP-1 cells with preimmune rabbit serum.

Lane 5: Immunoblot of COS-7 cells [transfected with Tiam1cDNA (FL1591)] with preimmune rabbit serum.

Lane 6: Immunoblot of COS-7 cells [transfected with the N-terminally truncated C1199 Tiam1cDNA] with preimmune rabbit serum.

FIGURE 2



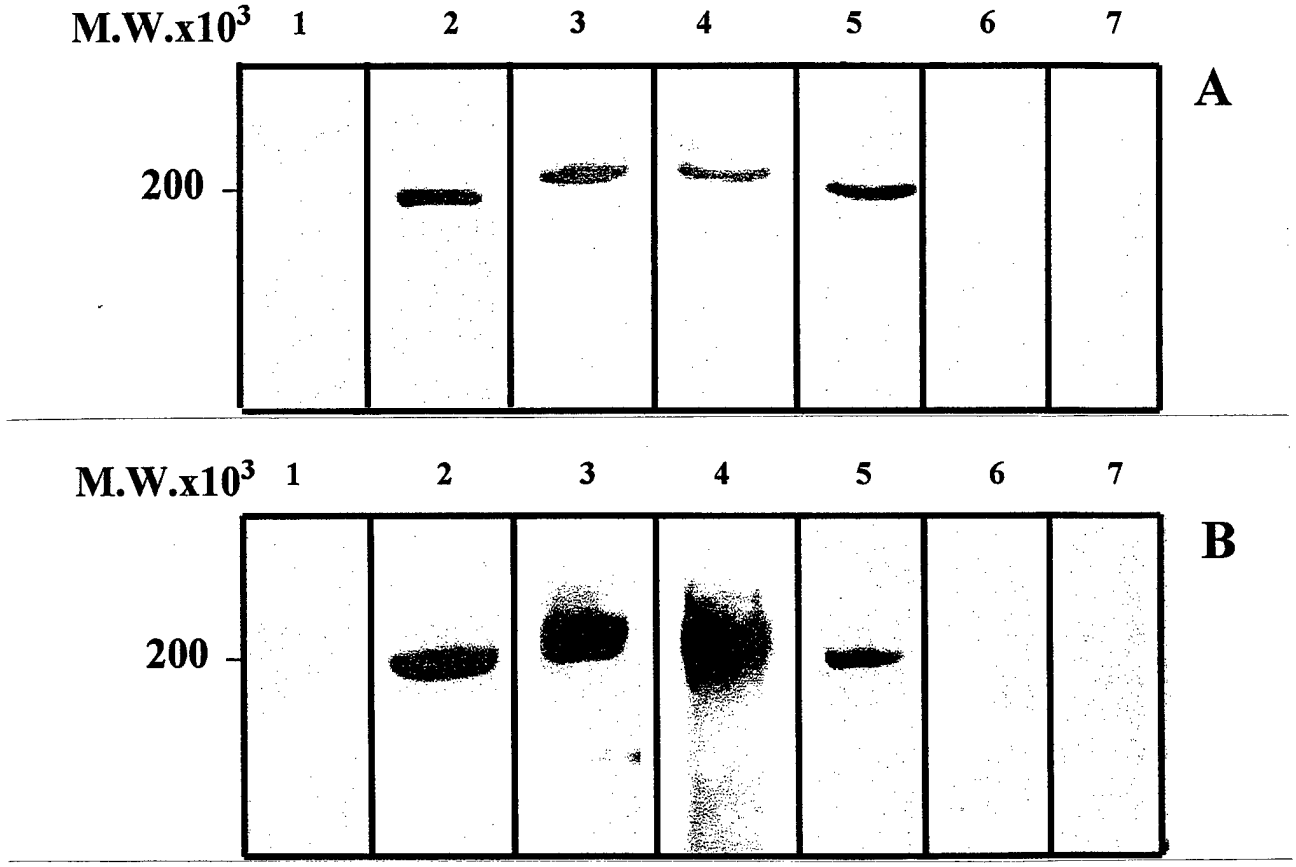
Tiam1-mediated GDP/GTP exchange for RhoGTPases.

Purified E. coli-derived GST-tagged GTPases (e.g. Rac1, Cdc42 or RhoA) were preloaded with GDP. First, 2pmole Tiam1 isolated from SP1 cells or COS-7 transfectants was added to the reaction buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 10mM $MgCl_2$, 100uM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 2.5μM GTPγ^{35}S (≈1,250Ci/mmol). Subsequently, 2.5pmole GDP-loaded GST-tagged RhoGTPases (e.g. Rac1, Rac1, Cdc42 or GST alone) were mixed with the reaction buffer containing Tiam1 and GTPγ^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in the Materials and Methods. The termination reactions were then filtered immediately through nitrocellulaose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTPγ^{35}S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of RhoGTPases (e.g. Rac1, Cdc42 or RhoA) was subtracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

A: Kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 (a), GST-Cdc42 (b) or GST-RhoA (c) or GST alone (d) in the presence of Tiam1 (isolated from SP-1 cells).

B: The maximal level of GTPγ^{35}S bound to GST-Rac1 in the presence of Tiam1 isolated from SP1 grown in 5% fetal calf serum (a-shaded bar) or 20% fetal calf serum (a-blank bar); or the full-length Tiam1 (1591) isolated from COS-7 transfectants grown in 5% fetal calf serum (b-shaded bar) or 20% fetal calf serum (b-blank bar); or the C1199 Tiam1 isolated from COS-7 transfectants grown in 5% fetal calf serum (c-shaded bar) or 20% fetal calf serum (c-blank bar); or Tiam1 isolated from vector-transfected COS-7 cells grown in 5% fetal calf serum (d-shaded bar) or 20% fetal calf serum (d-blank bar).

FIGURE 3



Detection of Tiam1-ankyrin complex in SP1 cells.

SP1 cells (5 x 10^5 cells) were solubilized by 1% Nonidet P-40 (NP-40) buffer and processed for anti-ankyrin or anti-Tiam1-mediated immunoprecipitation followed by immunblotting with anti-Tiam1 or anti-ANK1 (or anti-ANK3) antibody, respectively as described in the Materials and Methods.

A: Analysis of Tiam1-ANK1 complex:

Lane 1: Anti-ANK1-mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum.

Lane 2-3: Detection of Tiam1 in the complex by mouse anti-ANK1-mediated immunoprecipitation followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or rebloting with mouse anti-ANK-1 antibody (lane 3).

Lane 4-7: Detection of ANK1 in the complex by rabbit anti-Tiam1-mediated immunoprecipitation followed by immunoblotting with mouse anti-ANK1 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5); or peroxidase conjugated normal mouse IgG (lane 6) or peroxidase conjugated rabbit preimmune IgG (lane 7).

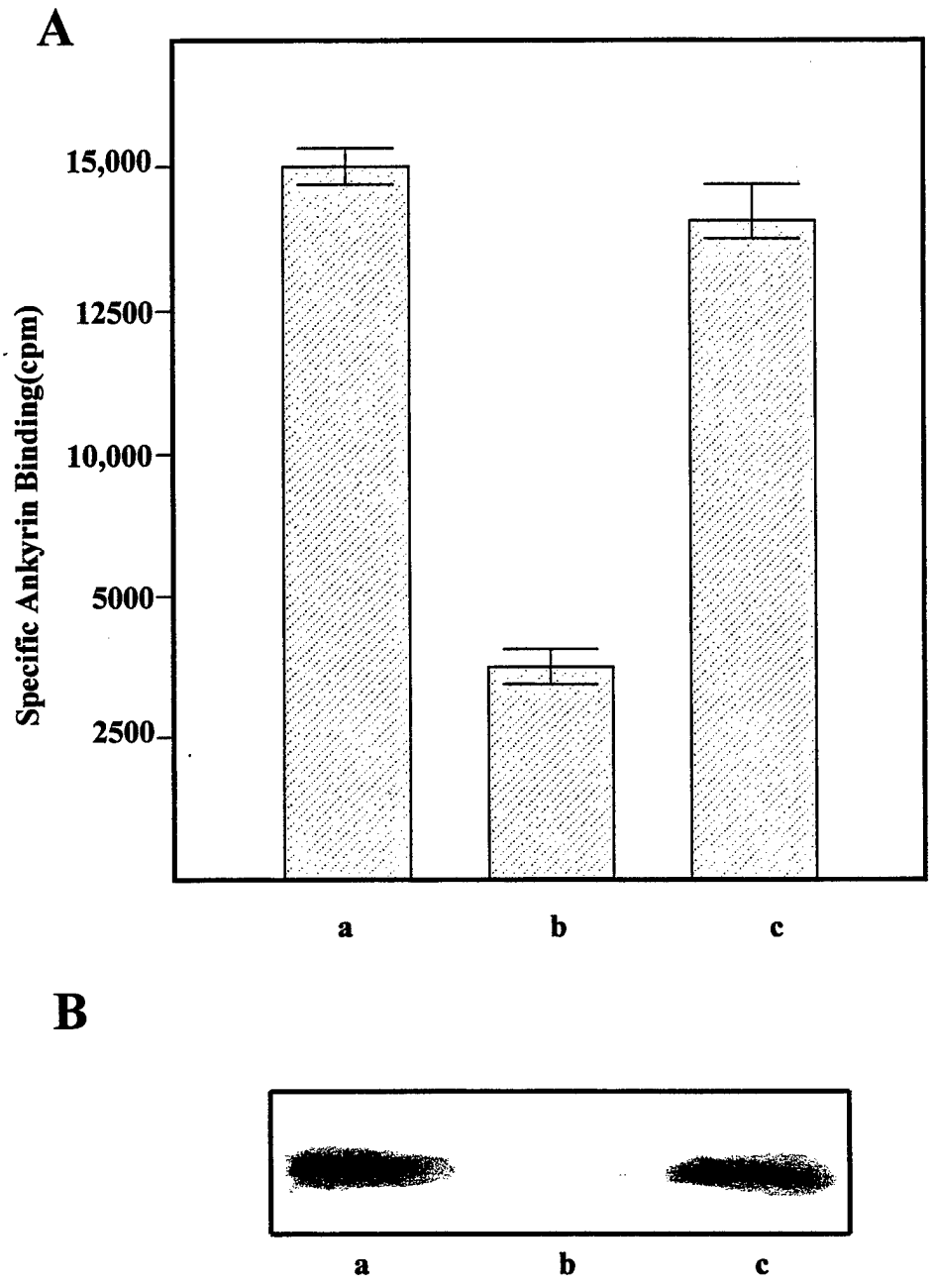
B: Analysis of Tiam1-ANK3 complex:

Lane 1: Anti-ANK3-mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum.

Lane 2-3: Detection of Tiam1 in the complex by mouse anti-ANK3-mediated immunoprecipitation followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or rebloting with mouse anti-ANK-3 antibody (lane 3).

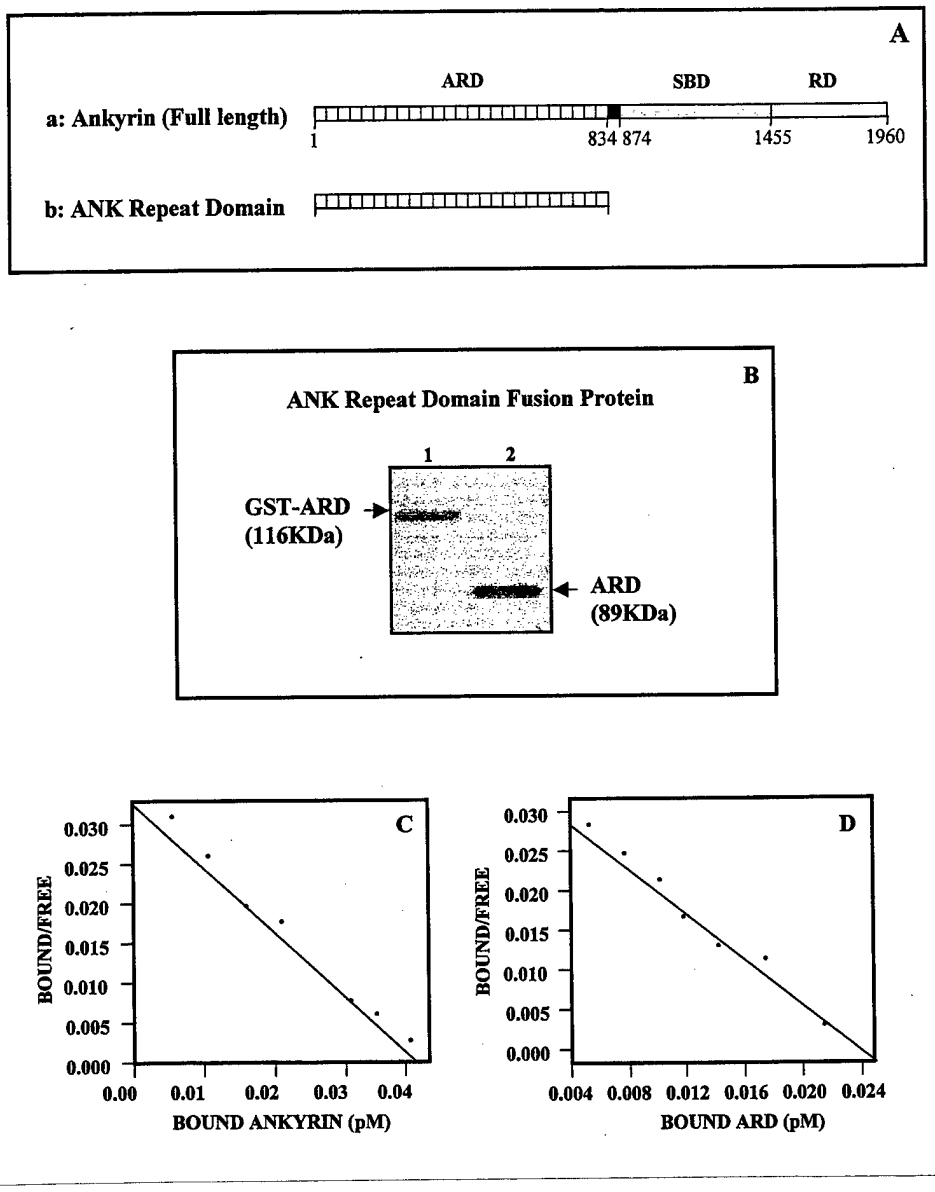
Lane 4-7: Detection of ANK3 in the complex by rabbit anti-Tiam1-mediated immunoprecipitation followed by immunoblotting with mouse anti-ANK3 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5); or peroxidase conjugated normal mouse IgG (lane 6) or peroxidase conjugated rabbit preimmune IgG (lane 7).

FIGURE 4



Binding interaction between Tiam1 and the cytoskeletal protein, ankyrin.
A: Tiam1 (isolated from SP-1 cells) bound to the anti-Tiam1 immuno-beads were incubated with ^{125}I-labeled ankyrin (5,000 cpm/ng protein) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c). Following binding, the immunobeads were washed extensively in binding buffer and the bead-bound radioactivity was estimated.
B: Autoradiogram of ^{125}I-labeled ankyrin binding to a polyacrylamide gel containing purified Tiam1 (isolated from SP-1 cells) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c).

FIGURE 5



Ankyrin structure and ankyrin repeat domain (ARD) fusion protein.

A-a: Schematic illustration of functional domains in ankyrin full length: ankyrin repeat domain (ARD), spectrin binding domain (SBD) and regulatory domain (RD).

A-b: ARD cDNA was constructed according to the strategy described in the Materials and Methods. This ARD cDNA construct encodes for the N-terminal region of the ankyrin membrane binding domain with a tandem array of 24 ankyrin repeats.

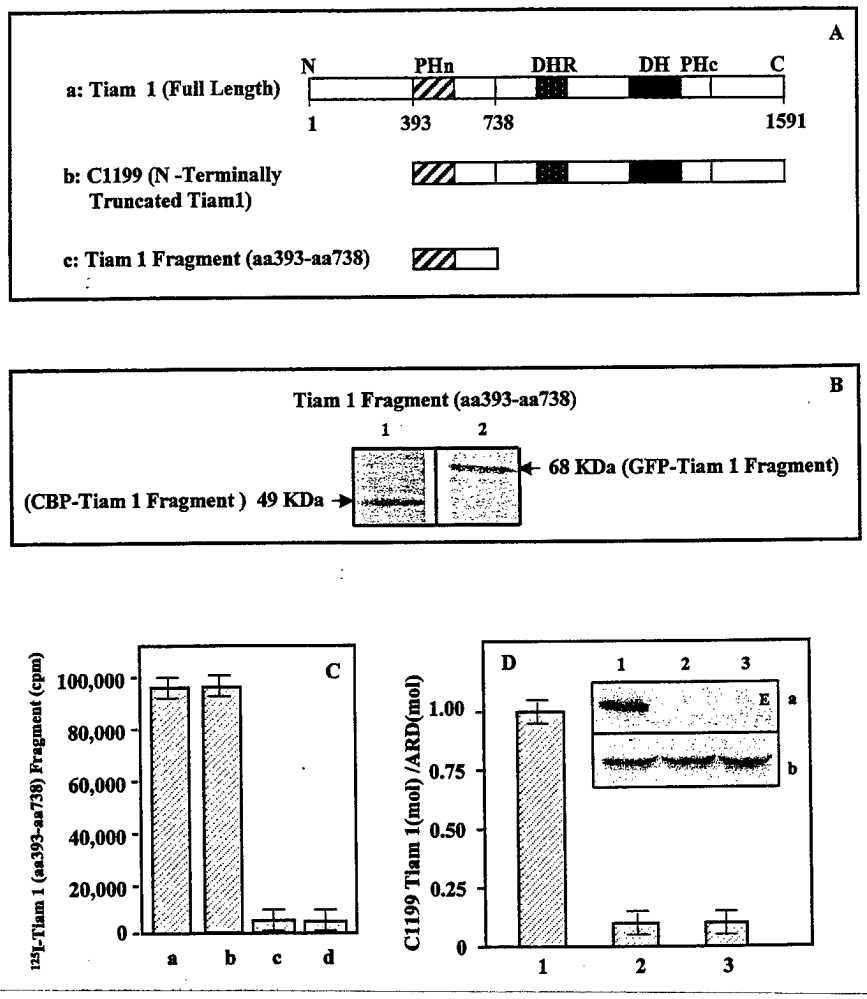
B: A coomassie blue staining of the 116kDa GST-ARD fusion protein purified by affinity column chromatography (lane 1); and the 89kDa ARD (lane 2) after the removal of GST by thrombin digestion.

C and D: Scatchard plot analyses of the equilibrium binding between ^{125}I-labeled ankyrin and Tiam1.

Various concentrations of ^{125}I-labeled ankyrin [e.g. intact erythrocyte ankyrin (ANK1) or ARD] were incubated with purified Tiam1-coupled beads at 4°C for 4 h. Following binding, beads were washed extensively in binding buffer and the beads-bound radioactivity was counted. As a control, ^{125}I-labeled ankyrin or ^{125}I-labeled ARD was also incubated with uncoated beads to determine the binding observed due to the non-specific binding of various ligands. Non-specific binding which represented approximately 20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. The values expressed in the result section represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled intact erythrocyte ankyrin (ANK1) and Tiam1 (C); and Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled ARD and Tiam1 (D).

FIGURE 6



Properties of Tiam1 and Tiam1 mutant proteins.

A-a: The full length Tiam1 contains DH, dbl-homology domain; DHR, discs-large homology domain; two pleckstrin homology (PH) domains [including the NH2-terminal PH (PHn) and the COOH-terminal PH (PHc)].

A-b: The N-terminally truncated C1199 Tiam1 encodes the C-terminal 1199 amino acids.

A-c: The Tiam1 fragment encodes the sequence between aa393-aa738.

B: Characterization of Tiam1 fragment (aa393-aa738) fusion proteins.

Coomassie blue staining of E. coli-derived CBP-Tiam1 fragment fusion protein purified by calmodulin affinity column chromatography (lane 1); and GFP-tagged Tiam1 fragment fusion purified by anti-GFP-conjugated affinity column chromatography (lane 2).

C-a: Binding of ^{125}I-Tiam1 fragment to ankyrin.

C-b: Binding of ^{125}I-Tiam1 fragment to ARD.

C-c: Binding of ^{125}I-Tiam1 fragment to ankyrin's spectrin binding domain.

C-d: Binding of ^{125}I-Tiam1 fragment to spectrin.

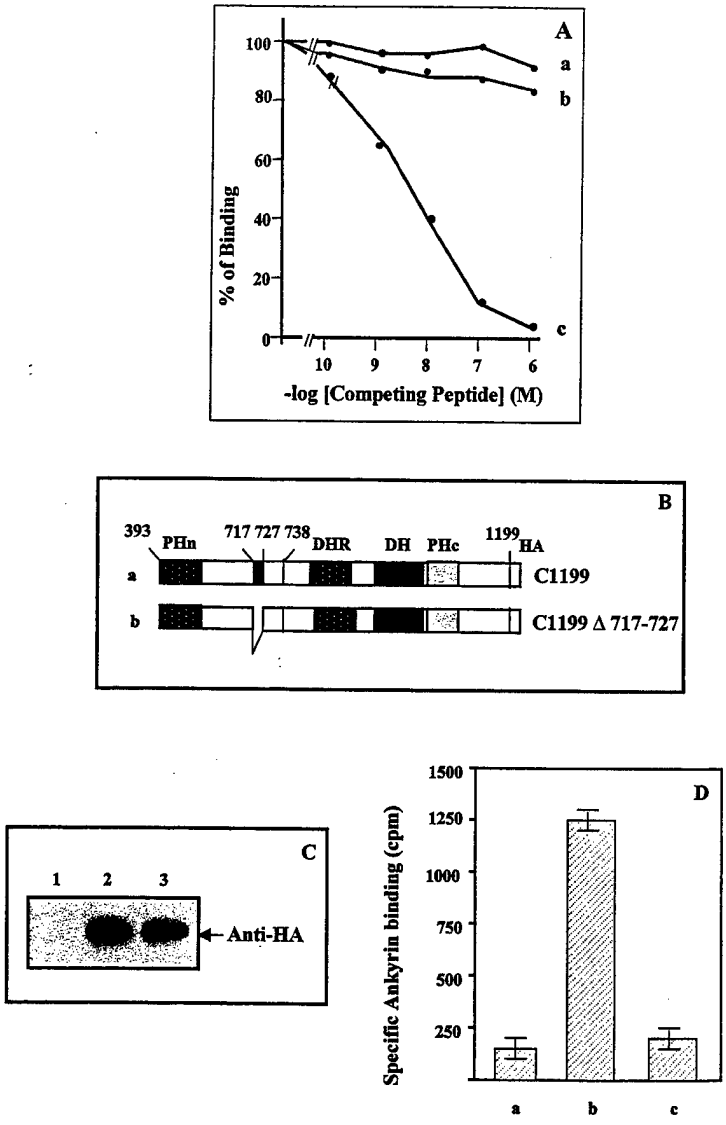
D and E: Binding analysis between GST-ARD and the recombinant C1199 Tiam1 in vitro.

In each reaction, 15-60 μl of glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in 0.5 ml of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100]. Purified C1199 Tiam1 (0.5μg-1.0μg) was then added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100μg) at 4°C for 4 h. Following binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150μl of 50mM Tris-HCl (pH 8.0) buffer containing 30mM glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was then determined by SDS-PAGE and Coomassie blue staining followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199Tiam1 (mol) was then calculated. Values represent relative binding abilities averaged from three experiments ±SEM.

D: The amount of C1199Tiam1 (mol) associated with GST-ARD (mol) was measured in the absence (lane 1) or the presence of recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3) using SDS-PAGE and Coomassie blue staining followed by densitometric analyses.

E: Coomassie blue staining of C1199Tiam1 associated with GST-ARD in the absence (lane 1) or the presence of recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3).

FIGURE 7



Identification of the ankyrin binding domain of Tiam1.

A: ^{125}I-labeled Tiam1 was incubated with ankyrin-coated beads in the presence of various concentrations of unlabeled synthetic peptide (GEGTDAVKRSL-corresponding to the sequence between aa717-aa727 of Tiam1) (c) or the scrambled sequence (GRATLEGSDKV) (a) or another Tiam1-related peptide (GTIKRAPFLGP) (corresponding to the sequence between aa399 and aa409 of Tiam1 (b) as described in the Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

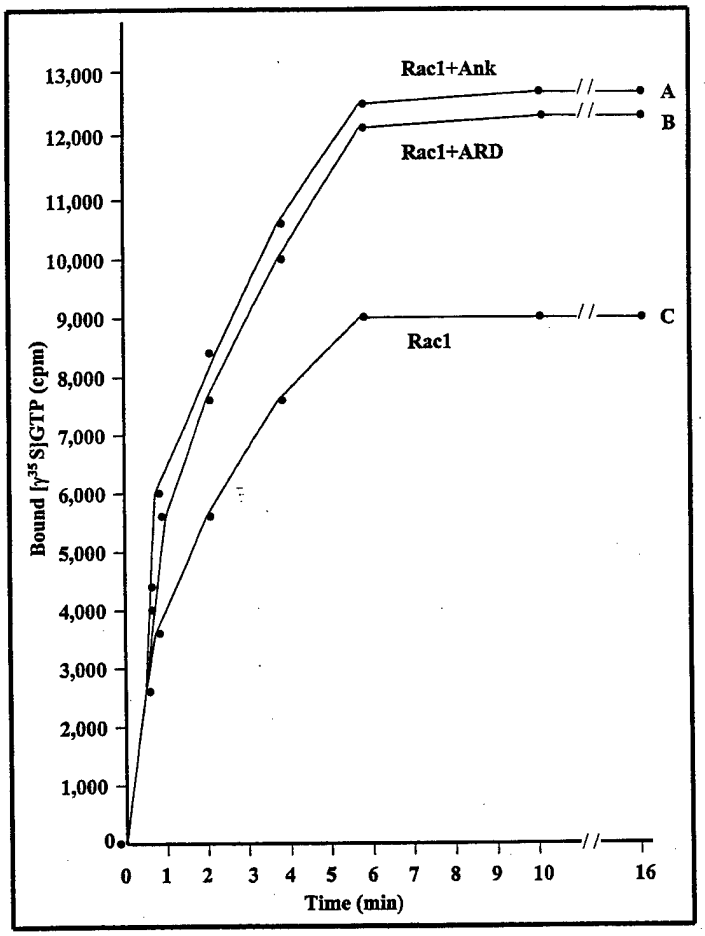
B: Schematic illustration of the in vitro mutagenesis approach used in this study.

Both C1199 Tiam1 (a) and C1199Tiam1Δ717-727 (lacking the sequence between aa717-aa727) (b) were constructed according to the strategy described in the Materials and Methods.

C: Anti-HA-mediated Immunoblot of SP-1 cells transiently transfected with vector alone (lane 1) or HA-tagged C1199 Tiam1cDNA (lane 2) or HA-tagged C1199Tiam1Δ717-727cDNA (lane 3).

D: The amount of ^{125}I-ankyrin binding to anti-HA immunoprecipitated materials isolated from SP-1 cells transfected with vector alone (a), or HA-tagged C1199Tiam1cDNA (b) or HA-tagged C1199Tiam1Δ717-727cDNA (c).

FIGURE 8

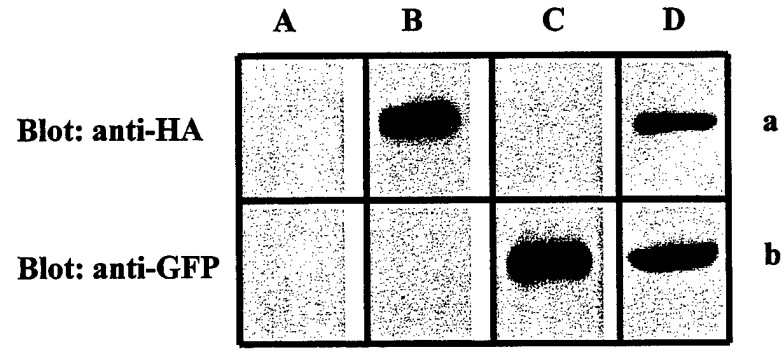


Stimulation of Tiam1-catalysed GDP/GTP exchange activity by ankyrin.

Purified E. coli-derived GST-tagged GTPases (e.g. Rac1, Cdc42 or RhoA) was preloaded with GDP. Subsequently, 2pmole of Tiam1 [isolated from untransfected or transfected cells according to the procedures described above] was preincubated with no ankyrin or ankyrin [e.g. intact ankyrin or ARD (1μg/ml)] followed by adding to the reaction buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 10mM $MgCl_2$, 100uM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 2.5μM GTPγ^{35}S (≈1,250Ci/mmol). Subsequently, 2.5pmole GDP-loaded GST-tagged RhoGTPases (e.g. Rac1, Rac1 or Cdc42) were mixed with the reaction buffer containing Tiam1 and GTPγ^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, and 10 mM $MgCl_2$ as described in the Materials and Methods. The termination reactions were then filtered immediately through nitrocellulaose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTPγ^{35}S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of RhoGTPases (e.g. Rac1, Cdc42 or RhoA) was subtracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

A-C: Kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 by Tiam1 (isolated from SP-1 cells) in the absence (C) or in the presence of ankyrin [e.g. intact erythrocyte ankyrin (ANK1) (A) or ARD fragment (B)].

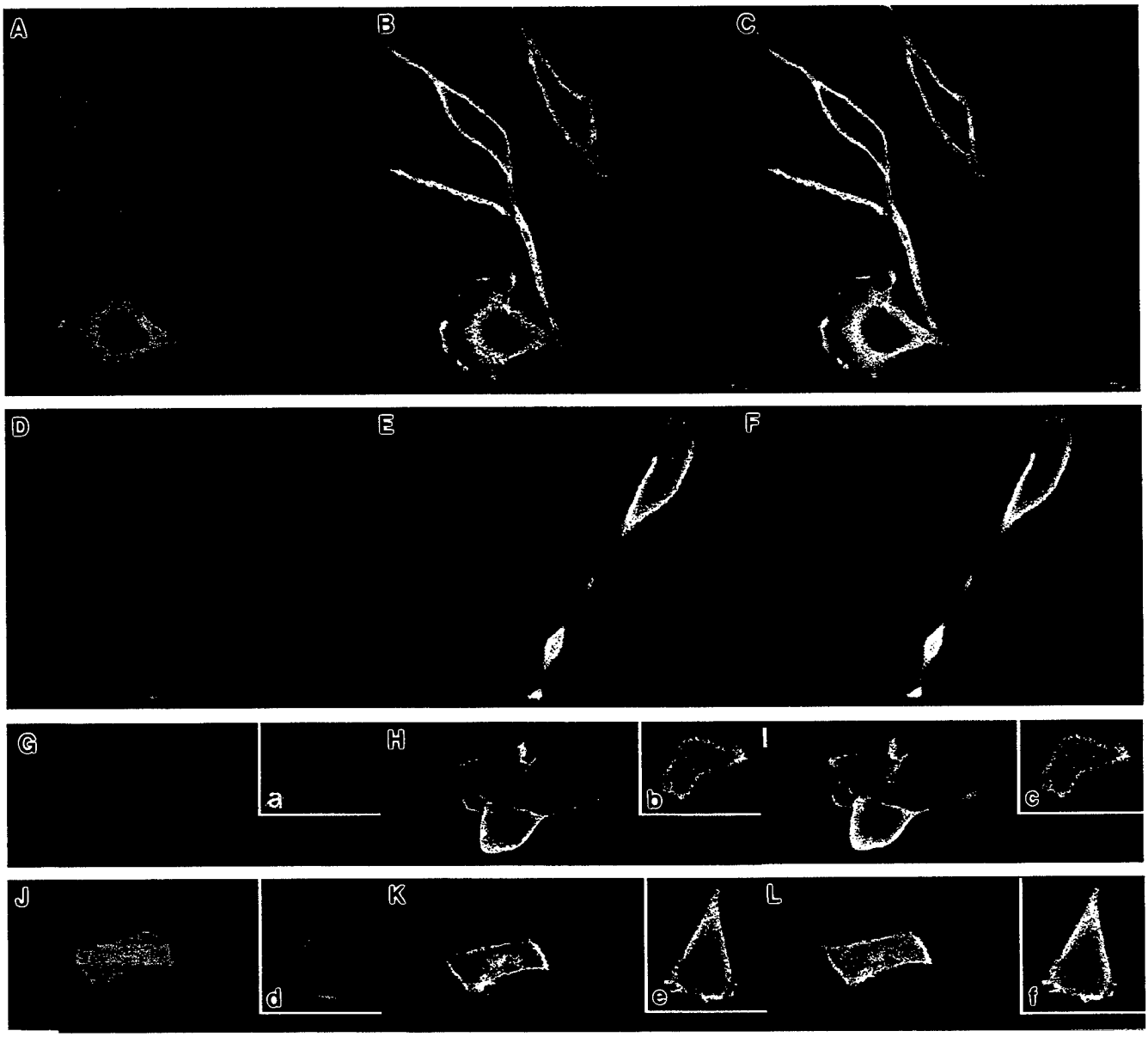
FIGURE 9



Transfection of SP1cells with HA-tagged C1199Tiam1cDNA(A) or GFP-tagged Tiam1 fragment cDNA (B) or co-transfection of HA-tagged C1199Tiam1cDNA and GFP-tagged Tiam1 frgament cDNA (C).

Detection of C1199 Tiam1 expression by anti-HA-mediated immunoblot in HA-tagged C1199 Tiam1cDNA transfected cells (lane B-a) or in vector-transfected cells (lane A-a); Detection of Tiam1 fragment expression by anti-GFP-mediated immunoblot in GFP-tagged Tiam1 fragment cDNA transfected cells (lane C-b) or vector-transfected cells (lane A-b); Detection of co-expression of C1199 Tiam1 and Tiam1 fragment by immunoblotting of cells (co-transfected with HA-tagged C1199 Tiam1cDNA and GFP-tagged Tiam1 fragment cDNA) with anti-HA antibody (D-a) and anti-GFP antibody (D-b), respectively; In controls, no signal was detected in HA-tagged C1199 Tiam1cDNA transfected cells or GFP-tagged Tiam1 fragment cDNA transfected cells using anti-GFP (lane B-b) or anti-HA (lane C-a)-mediated immunoblotting , respectively.

FIGUER 10



Double immunofluorescence staining of ankyrin and Tiam1 cDNA (e.g. C1199 Tiam1 cDNA or Tiam1 fragment cDNA)-transfected SP1 cells.

SP1 cells (transfected with HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or co-transfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA) were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immuno-reagents as described in the Materials and Methods.

A, B and C: Rh-labeled anti-ANK3 staining (A), FITC-anti-Tiam1 staining (B) and co-localization of ankyrin and Tiam1 (C) in untransfected SP1 cells.

D, E and F:Rh-labeled anti-ANK3 staining (D), FITC-anti-HA-labeled C1199 Tiam1 staining (E), and co-localization of ankyrin and C1199 Tiam1 (F) in HA-tagged C1199 Tiam1 cDNA transfected SP1 cells.

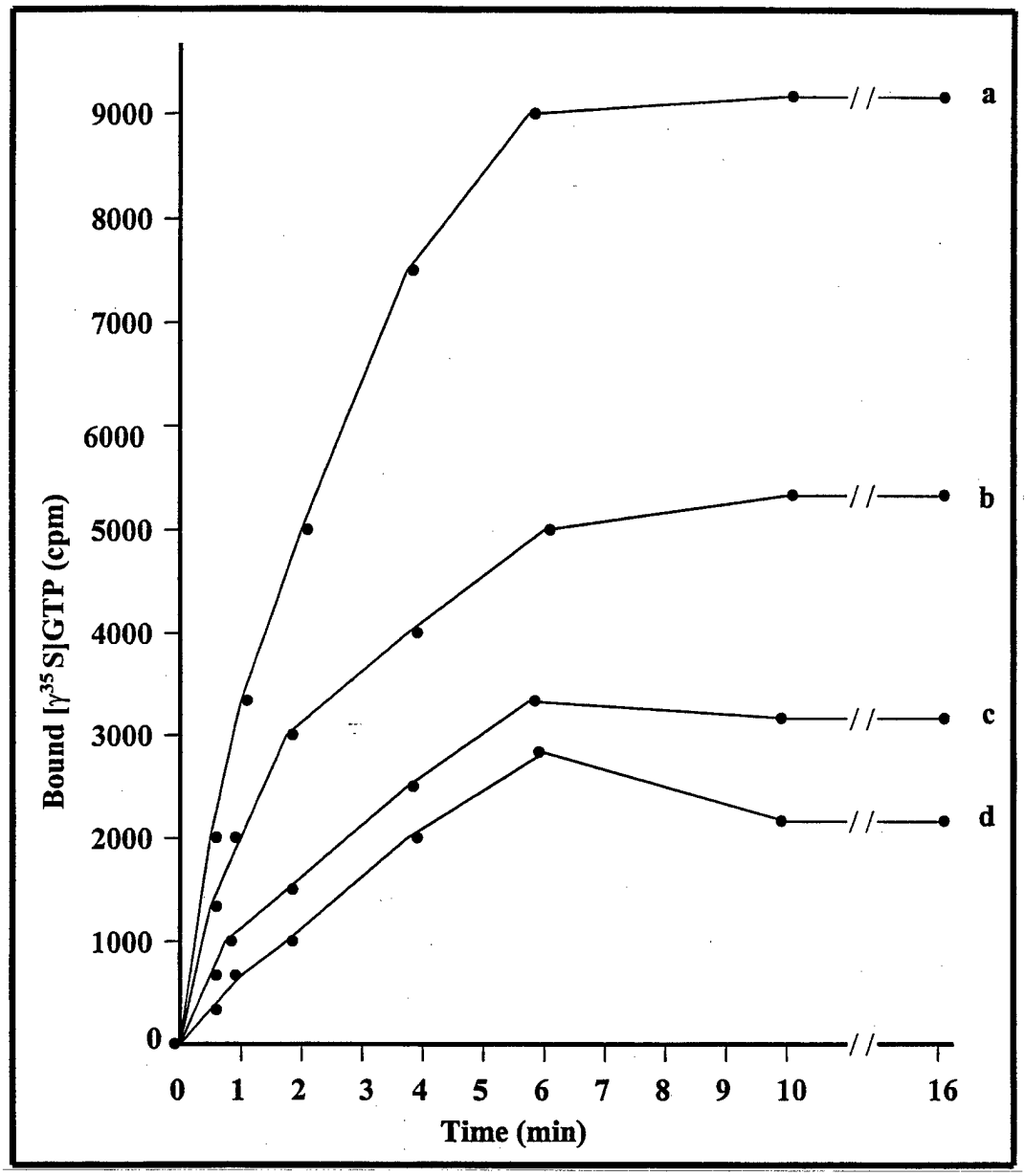
G, H and I: Rh-labeled anti-ANK3 staining (G), GFP-tagged Tiam1 fragment (H) and co-localization of ankyrin and Tiam1 fragment (I) in GFP-tagged Tiam1 fragment cDNA transfected SP1 cells.

a, b and c:Rh-labeled preimmune IgG staining (a), GFP-tagged Tiam1 fragment (b) and co-localization of preimmune IgG and Tiam1 fragment (c) in GFP-tagged Tiam1 fragment cDNA transfected SP1 cells.

J, K and L: Rh-labeled anti-HA staining of C1199 Tiam1 (J), GFP-tagged Tiam1 fragment (K) and co-localization of C1199 and Tiam1 fragment (L) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA.

d, e and f: Rh-labeled anti-ANK3 staining (d), GFP-tagged Tiam1 fragment (e) and co-localization of ankyrin and Tiam1 fragment (f) in SP1 cells co-transfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA.

FIGUER 11



Kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 in the presence of ankyrin-associated Tiam1 isolated from SP-1 cells [transfected with HA-tagged C1199 Tiam1 cDNA (a) or GFP-tagged Tiam1 fragment cDNA (d) or co-transfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (c) or vector alone (b)].

Purified E. coli-derived GST-tagged GTPases (e.g. Rac1, Cdc42 or RhoA) were preloaded with GDP. First, 2pmole ankyrin-associated Tiam1 isolated from various SP1 transfectants was added to the reaction buffer containing 20mM Tris-HCl (pH 8.0), 100mM NaCl, 10mM $MgCl_2$, 100uM AMP-PNP, 0.5 mg/ml bovine serum albumin, and 2.5μM GTPγ^{35}S (≈1,250Ci/mmol). Subsequently, 2.5pmole GDP-loaded GST-tagged RhoGTPases (e.g. Rac1, Rac1, Cdc42 or GST alone) were mixed with the reaction buffer containing ankyrin-associated Tiam1 and GTPγ^{35}S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in the Materials and Methods. The termination reactions were then filtered immediately through nitrocellulaose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTPγ^{35}S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of RhoGTPases (e.g. Rac1, Cdc42 or RhoA) was subtracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

BINDING: We have prepared the report according to the instruction provided by DOD.

FINAL REPORTS:

Paper Publications:

1. Iida, N. and Lilly Y.W. Bourguignon. Coexpression of CD44 Variant (v10/ex 14) and CD44s in Human Mammary Epithelial Cells Promotes Tumorinogenesis. J. Cell Physiol. 171:152-160 (1997).

2. Bourguignon, Lilly Y.W., H.B. Zhu, A. Chu, L. Zhang and Mien-Chie Hung. Interaction Between the Adhesion Receptor, CD44 and the Oncogene Product, $p185^{HER2,}$ Pomotes Human Ovarian Tumor Cell Activation. J. Biol. Chem. 272:27913-27918 (1997).

3. Bourguignon, Lilly Y.W., Role of Membrane-Associated Cytoskeleton In Regulating Immune Cell Function Membrane Skeleton Linker Proteins In Immune Cells. In "Encyclopedia of Immunology". (I.M. Roitt and P.J. Delves, eds.), Academic Press, (1997)(Invited Review Article).

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8. Kalish, E., N. Iida, F.L. Moffat and Lilly Y. W. Bourguignon. A New CD44v3-Containing Isoform Is Involved in Tumor Cell Migration and Human Breast Cancer Progression. Front Biosci. 4:1-8 (1999).

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12. Zhu, D. and Lilly Y.W. Bourguignon. Interaction Between CD44 and The Repeat Domain of Ankyrin Promotes Hyaluronic Acid (HA)-Mediated Ovarian Tumor Cell Migration. J. Cell Physiol. 183:182-195 (2000).

13. Diaz, F. and Lilly Y.W. Bourguignon. Selective Down-Regulation of IP3 Receptor Subtypes By Caspases and Calpain During TNFα-Induced Apoptosis of Human T-Lymphoblasts. (Cell Calcium, In Press, 2000).

14. Bourguignon, Lilly Y.W., H.Zhu, L. Shao and Y.W.Chen, Ankyrin-Tiam1 Interaction Promotes Rac1 Signaling and Metastatic Breast Tumor Cell Invasion and Migration. (J. Cell Biol., In Press, 2000).

Abstracts:

1. Dan Zhu and Lilly Y. W. Bourguignon. The Ankyrin-Binding Domain Of CD44s Is Required For Cell Adhesion, Src Kinase Association And Proliferation In Human Prostate Tumor Cells. Proceeding of the American Association for Cancer Res. 38:532 (1997).

2. Bourguignon, Lilly Y. W., Z. Gunja-Smith, N. Iida, H. B. Zhu, L.J.T. Young and R. D. Cardiff. CD44v3 Is Responsible For Vascular Growth Factor (VEFG) Binding, Membrnae-Cytoskeleton Interaction, and Matrix Matalloproteinase (MMP) Activation In Metastatic Breast Tumor Cells. Proceeding of the American Association for Cancer Res. 38:532 (1997).

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